HIGH FIBER LOW ENERGY DIET FOR MOLT INDUCTION IN LAYING HENS: THE IMPACT OF ALFALFA ON PHYSIOLOGY, IMMUNOLOGY AND BEHAVIOR

A Dissertation

by

CLAUDIA SHARENE DUNKLEY

Submitted to the Office of Graduate Studies of
Texas A&M University
in partial fulfillment of the requirements for the degree of

DOCTOR OF PHILOSOPHY

December 2006

Major Subject: Poultry Science

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ABSTRACT

High Fiber Low Energy Diet for Molt Induction in Laying Hens: The Impact of Alfalfa on Physiology, Immunology and Behavior.

(December 2006)

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Feed withdrawal is commonly used by commercial egg producers to induce molt and stimulate multiple egg-laying cycles in their flocks. However, the practice can compromise the welfare of the birds by elevating stress, suppressing the immune response and causing abnormal behavior. An alternate molt diet was examined using alfalfa diets, and a series of experiments were conducted to evaluate the physiological, immunological and behavioral responses of laying hens fed these diets. We assessed the changes in the levels of blood plasma metabolites after hens were challenged with 10^6 colony forming units of *Salmonella* Enteritidis (SE). Hens fed alfalfa diets displayed similar ($P \geq 0.05$) levels of cholesterol, glucose, and total protein when compared to full-fed hens. Reduced levels ($P \leq 0.05$) of triglycerides were observed in the alfalfa fed and feed withdrawal hens when compared to the full-fed hens. The immune responses of SE challenged laying hens fed alfalfa diets showed similar ($P \geq 0.05$) heterophil to lymphocyte ratios (H: L) to full-fed hens whereas the feed withdrawal hens displayed

elevated ($P \leq 0.05$) H: L ratios. The feed withdrawal hens displayed reduced ($P \leq 0.05$) levels of serum IgY early in the trial when compared to the alfalfa and full-fed hens. The alfalfa fed hens displayed lower levels of acid α glycol protein than the feed withdrawal hens and higher levels than the full-fed early during the trial but returned to levels that were similar ($P \geq 0.05$) to the full-fed hens. The hens fed alfalfa diets displayed elevated non-nutritive pecking behavior early in the trial however, this behavior declined to levels similar ($P \geq 0.05$) to the full-fed hens by the end of the trial. No differences in aggressive behavior were observed between the alfalfa fed hens and the full-fed hens. Hafnium chloride did not effectively mark the alfalfa diet; however, it can be used to track the passage of corn-soy layer ration. This research suggests that the application of alfalfa as an alternative molt diet can be effective in reducing potentially harmful effects which are usually associated with feed withdrawal.

DEDICATION

I thank God almighty for carrying me through. I dedicate this dissertation to my sons, Kingsley Jr., Nicholas, Khori and Ricardo. You have all been there with me through the highs and the lows and have endured a lot in order for me to complete this journey. Kingsley Sr., my husband, we embarked on this journey together, for all the times I felt like giving up you were always there to encourage me, you are my partner in every way.

I also dedicate this dissertation to my numerous family members especially my parents, Clarence and Sylvia Powell, my Aunt Lucille, my brothers Duane and Douglas and sister in law, Karen, my Uncle Mushae and Aunt Carron; your constant encouragement, prayers and monetary support were invaluable.

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TABLE OF CONTENTS

CHAPTER Page

LIST OF FIGURES

FIGURE Page

FIGURE Page

LIST OF TABLES

CHAPTER I

INTRODUCTION

Birds undergo a series of molts during their life span and will change at least four different plumages from hatching to their first annual cycle (Lucas and Stettenheim, 1972). At the end of a laying cycle egg production and quality decline significantly, for commercial egg producers this will mean a period of unproductivity because the molt is incomplete in commercial laying hens and they continue to lay eggs at low rates for a prolonged period of time (Berry, 2003). Historically, commercial egg producers in the US will seek to obtain a more uniform molt within the flock and they have used feed removal as a means of inducing a molt (Bell, 2003). Conventional feed withdrawal programs involves the removal of feed, water (no more than 3 days) or both from the hens and also reducing the photo-period in the houses to 8 h lighted period or less (Bell, 2003). After the induced molt, egg production and quality improve significantly compared to the pre-molt period. Commercial egg producers in the US typically implement induced molt programs when laying hens are about 65 wk of age so that the birds can be kept through a second laying cycle. Feed withdrawal causes hens to cease egg production and results in the regression of the reproductive system. When the hens are stimulated to return to production, the rejuvenated tract produces eggs with better shell and interior quality and at a higher rate than had been the case before they were molted (Webster, 2000).

This dissertation follows the style and format of Poultry Science.

Induced molt by feed withdrawal compromise the birds' immune system making them susceptible to infection by a number of microorganisms (Holt, 2003; Ricke, 2003). Of particular interest is *Salmonella enterica* serovar Enteritidis (SE). USDA-Animal and Plant Health Inspection Service (2000) stated that there was a doubling of environmental SE numbers in molted versus non-molted flocks. *Salmonella* infection in laying hens is a serious food safety concern, since infection of a bird could lead to the production of SE positive eggs after molt (Humphrey et al., 1993). Among the investigated mechanisms that are known to cause decreased resistance include alterations in the intestinal microflora (Corrier et al., 1997; Holt and Porter, 1992; Savage, 1989), intestinal physiology (Barnes et al., 1979; Corrier et al., 1997; Duke, 1986; Holt, 1993), and host defenses (Ben-Nathan et al., 1981, 1977; Holt, 1992a, 1993). The practice of feed withdrawal has also been characterized as stressful and frustrating (Duncan and Wood-Gush, 1971). Dawkins (1999) stated that the two components of animal welfare are physiological and psychological health. While the physiological health of the birds can be determined based on physical appearance, the psychological health is more difficult to assess. Webster (2000) postulated that behavior of birds might give direct indication of the well being of hens undergoing feed withdrawal.

Increased concerns of reduced animal welfare during an induced molt by feed deprivation had prompted investigations into alternative methods. Attempts have been made to incorporate nutritional imbalances, ingredients that decrease appetite, addition of fillers or administration of hormones to induce molts (Bell, 2003; Holt 2003; Park et al., 2004) as means of inducing a molt. The incorporation of high fiber low energy diets

to induce a molt has been suggested because such diets are usually low in metabolizable energy and high fiber diets will give the animal a feeling of satiety resulting in reduced feed intake (Rijnen et al., 1999). Alfalfa has the potential to serve as a dietary source for inducing a molt, it is high in crude protein (17.5%) and crude fiber (24.1%) and has a low level of metabolizable energy of about 1200 kcal/kg when compared to layer ration which has a metabolizable energy of 2,965 kcal/kg (NRC, 1994). It has the slowest passage through the gastrointestinal tract of the chicken (Sibald, 1979, 1980) and high concentrations in the diet of laying hens decreases growth rate and reduce egg production (Heywang, 1950). Alfalfa diets can effectively induce molt resulting in a second laying cycle similar to hens molted by feed deprivation (Donalson et al., 2005) and reduce SE colonization and shedding in the gastrointestinal tract and internal organs of laying hens during an induced molt (Woodward et al., 2005; McReynolds et al., 2005, 2006). In order to complete the investigation into the potential of alfalfa as an alternate molt diet, it is necessary to examine the physiological and psychological effects of the diet in the laying hens during a molt. The general objectives of these experiments were first to evaluate the changes in the physiological changes in hens fed an alfalfa diet during an induced molt after they were infected with SE. Second to evaluate the immunological changes in hens fed an alfalfa diet during an induced molt after they were infected with SE. Third to assess the behavior of hens during an induced molt while they were fed different combinations of alfalfa diets. Lastly, to examine the passage of the alfalfa through the gastrointestinal tract of laying hens.

CHAPTER II

LITERATURE REVIEW

Molting in the Avian Species

Molting in the avian species involves the periodic shedding and replacement of feathers. It also involves the involution of the hens' reproductive system resulting in a reproductive quiescence. During their natural lifespan, birds will undergo a series of different molts. From hatching up to their first annual cycle, birds will change at least four different plumages including; natal down, juvenile, alternate and basic plumages (Lucas and Stettenheim, 1972). Generally speaking, many avian species will have two molts per year, a pre-nuptial or pre-alternate molt and a post-nuptial or post-alternate molt. The post-nuptial molt is associated with the reproductive quiescence. It is a more complete molt that follows a breeding or laying cycle and involves the loss of feathers from the wings, tail, and body of the bird. Molting is usually considered to be more than simply plumage replacement since it also involves physiological changes in the bird (Stettenheim, 1972). These physiological changes include vascularization of the feather follicles and papillae (Stettenheim, 1972), osteoporosis (Meister, 1951; Murphy, 1996). Fractional protein synthesis rate, not only for feather keratin synthesis rate, but primarily for skeletal muscle synthesis (Murphy and Taruscio, 1995; Murphy, 1996) are also changes that occur during molting. Others have reported changes in metabolic rate, reduction in body fat (Kuenzel and Helms, 1974), and increased heterophil: lymphocyte ratio (Gross and Siegel, 1983; Davis et al., 2000) during a molt.

The post-nuptial molt period is a critical period in the bird's cycle as it involves major remodeling of the body and also the resetting of the neural system in order to respond to photo-stimulation (Kuenzel, 2003). A variety hormones and neuropeptides are involved in the remodeling process. One function of vaso-active intestinal polypeptide is to shut down the reproductive system by initiating the incubation behavior such as broodiness which is usually followed by a post-nuptial molt (Kuenzel, 2003).

In wild birds, the primary initiating factor of molting is the establishment of broodiness. After the onset of this broodiness, the hen will undergo the involution of the reproductive system, followed by cessation of lay, and finally the shedding and replacement of feathers (Sherry et al., 1980). During this time, the bird will voluntary reduce their intake of food. This is known as "spontaneous anorexia" (Mrosovsky and Sherry, 1980). A hen can lose up to 20% of its body weight during spontaneous anorexia; one half of this lost weight is from the involution of the reproductive tract. After the chicks have hatched, the hens will once again begin to consume feed and the feathers will be replaced. This process is a natural phenomenon in species that live in environments where incubation and feeding is incompatible (Sherry et al., 1980).

Control of Reproduction in Chickens

Photoperiod

Most birds are essentially seasonal breeders especially in temperate and northern latitudes birds breed under the influence of lengthening days (Etches, 1996). Reproduction in birds is controlled by interactions with light schedule, feeding and stress. Changes in response to photoperiod are of fundamental importance to seasonal

cycles in avian reproductive development, they must be able to recognize the changes in day length in order to differentiate between long and short days. For this purpose, birds utilize internal circadian rhythms. The period during which a bird is sensitive to light is called the photosensitive phase and this predicts the effects of photo-schedules. The importance of light intensity in photoperiodic stimulation of gonadotropin secretion is unclear however in studies where light intensity was measured the rate of egg production was proportional to intensity between 0.2 and 5 lux (Sharp et al., 1987). Short days (SD; photophase ≤8 h) do not illuminate the photosensitive phase, however, long days (LD; photophase ≥13 h) illuminates it and subsequently initiates Luetinizing hormone (LH) secretion (Etches, 1996). In birds photo-stimulation induces an increase in plasma LH and also follicle-stimulating hormone (FSH) (Gooden and Scanes, 1977; Bacon and Long, 1995). In most vertebrates, the changing day length is perceived and transduced into activation of the hypothalamo- pituitary gonadotrophic (HPG) axis (Bentley et al., 2003; Follet, 1984; Nicholls et al., 1988). A stimulatory photo-schedule must transmit two signals to the hen; first she sets her circadian clock at dawn, eleven hours after the circadian clock starts it signals the photosensitive phase. The second signal is then transmitted during this phase when the exposure to light is registered by the hypothalamic photoreceptors (Etches, 1996).

After a period of 12 to 15 months of lay, the effects photo-periodism begins to decline and birds cannot maintain maximal levels of gonadotropin secretions. This is referred to as photorefractoriness and the development of absolute photorefractoriness usually leads to the termination of seasonal breeding in most birds. This is usually

followed by a post- nuptial molt (Follett, 1984; Nicholls, 1988). Photo refractoriness is considered to cause a molt or is at least necessary for it's induction (Farner et al.,1980). The dissipation of photorefractoriness, thus the re-setting of the hypothalamus can be obtained by inducing a molt, thereby making the hypothalamus able to receive and transmit photoperiodic signals at a higher level (Etches, 1996). Photorefractoriness, causes the GnRH cell bodies in the brain to shrink and the fibers emanating towards the median eminence (ME) to decrease (Foster et al., 1987; Goldsmith et al., 1989; Boulakoud and Goldsmith, 1991). As a result, LH and FHS release will be reduced (Dawson and Goldsmith, 1982, 1983) leading to gondal regression.

Role of the Hypothalamus

Light is perceived through photoreceptors that transduce energy contained in photons into biological signals. For reproduction in the avian species the perception of light does not depend on the photoreceptors in the eyes but on the photoreceptors in the hypothalamus. These photoreceptors are biological transducers that convert the photon energy into neuron energy which are amplified by the endocrine system to control the function of the gonads which in turn influence the reproductive function, behavior and secondary sexual characteristics (Etches, 1996). A reduction in GnRH neurons, are involved in the development of photo refractoriness (Bluhm et al., 1991; Hahn and Ball, 1995), this results in a decrease in gonadotropin secretions.

Gonadotropic function and synthesis are regulated by hypothalamic peptides. The release of reproductive the hormones is controlled by releasing hormones at the hypothalamo-pituitary-axis of the brain. Short-term feed restriction has shown effects on

the reproductive axis in laying hens. Verheyen et al. (1987) observed that acute feed restriction reduced plasma progesterone concentration. Due to a feedback mechanism, the reduction of these hormones can lead to a reduction in plasma concentration of luteinizing hormone (LH) and an increase in follicle stimulating hormone (FSH) (Johnson and vanTienhoven, 1980; Tanabe et al., 1981; Decuypere et al., 1992)

Prolactin (PRL) secretion is photo-induced and research has demonstrated that it play a major role in molting (Juhn and Harris, 1958; Millam and Halawani, 1986; Payne, 1972). Prolactin is anti-gonadotrophic/ gonadal in birds (Camper and Burke, 1977; Buntin et al., 1988) and therefore the increase in plasma PLR is involved in the development of photorefractoriness and the induction of post-nuptial molt. Vaso-active intestinal polypeptide (VIP) has been identified in the avian species as a PRL releasing hormone (Sharp et al., 1989; Mauro et al., 1989). Immunization against VIP is known to suppress photo-induced PRL secretion in turkeys (El Halawani et al., 1996). Dawson and Sharpe (1998) concluded that it is possible that VIP can control gonadal regression, hence molt, either directly through PRL or by some other mechanisms.

One of the most important regulatory peptide in the avian hypothalamus is chicken Luteinizing Hormone- Releasing Hormone (cLHRH). Two types have been observed, cLHRH-I and cLHRH-II. Chicken Luteinizing Hormone- Releasing Hormone I (cLHRH-I) was first isolated from the chicken hypothalamic tissue by King and Milar (1982). The presence of cLHRH-I immunoreactive (ir) neurons throughout the septal pre-optic area and the anterior hypothalamus, have been observed in several avian studies (Millam et al., 1993; Van Gils et al., 1993). Most of these neurons projected to

the ME and it is here that they release their peptide contents into the hypophysial portal blood system, thereby stimulating the release of LH and FSH from the pituitary. These gonadotropes further stimulate the release of steroid hormones such as estrogen and progesterone, from the gonads which in turn establish a positive feedback mechanism to the hypothalamus. The functionality of the cLHRH system is estrogen-dependent. The release of cLHRH in both mammalian and avian species is regulated by several neuromodulators and neuropeptides (Leranth et al., 1988; Merchenthaler et al., 1990). These neuropeptides regulate the release of cLHRH at the terminals in the ME (Contijoch et al., 1992). Arginine vasotocin (AVT) has been considered as a factor in the regulation of cLHRH-I release in birds. Localization of AVT in structures throughout the central nervous system of chicken has been revealed by immunochemical studies (Tennyson et al., 1986). D'Hondt and others (2000) postulated that AVT should be considered, as an important regulator of cLHRH-I release, and hence of reproductive function since there is co-localization of LHRH-I and AVT in neurons throughout the pre-optic hypothalamic area.

Several *in vivo* and *in vitro* studies have shown that both peptides are equally potent in releasing LH and FSH (King et al., 1988; Millar and King, 1984; Sharp et al., 1987; Wilson et al., 1989). Sharp and others (1987) used hens and cockerels to compare the response in LH plasma levels after i.v. injection with cLHRH-I or cLHRH-II. They found that in hens, cLHRH-II was more effective in releasing LH than cLHRH-I. In a later study Sharp et al. (1989) observed cLHRH-II in laying hens, occurred at low levels in the brain and they also noted that cLHRH-II could not be detected in the ME whereas,

cLHRH-I occurred in abundance. In the same study they observed that immunization against cLHRH-I and not cLHRH-II, resulted in a complete regression of the reproductive system and a sharp decline in the plasma LH concentration. These results suggested that the secretion of gonadotropins in hens was regulated mostly, if not exclusively by cLHRH-I and not cLHRH-II.

Molting in the Commercial Egg Industry

Economic Factors

Domestic hens like most species of wild birds experience a naturally occurring molt. This however, is usually incomplete and hens continue to lay eggs at low rates for a prolonged period of time (North and Bell, 1990). This usually indicates the end of the useful life of a flock, which for a one-cycle (non-molted) program is approximately 80 weeks old (Bell, 2003). At the first sign of molting commercial egg producers typically sell these hens and replace them with a new flock. Egg producers usually make the decision to molt hens for a second laying cycle based on the cost of eggs which is approximately one cent more per dozen in the second cycle and the cost of feed and replacement pullets (North and Bell, 1990). Replacement pullets are the most expensive costs for a layer operation, therefore, commercial egg producers seek to extend the productive laying life of their flock for as long as it is profitable (Parkhurst and Mountney, 1988), therefore an induced molt is implemented as a part of a program to optimize the use of replacement pullets on layer farms (Bell, 2003). However, if replacement pullets are inexpensive, or if egg prices are high and the farmer have egg

production quotas, there will then be an incentive to produce at higher prices and molting will then not be profitable (Bell, 2003).

Another economic factor that can influence the decision to molt is the declining value of Leghorn hens carcasses and the also the declining number of processors willing to buy them (Webster, 1995; McDaniels and Aske, 2000). It is estimated that a program that includes molting will result in at least 30% higher profit margins than an all pullet program (Berry, 2003). It has become an industry wide practice and it contributes significantly to the commercial industry profits. Based upon a $0.65 per year per hen housed in a two cycle program compared to $0.50 in a one cycle program the curtailment of such a practice and a return to an all-pullet program would result in the requirement of 47% more chicks per year and the removal of 47% more spent hens each year (Berry, 2003).

Management of Artificial Molt Induction

Over the years, researchers have developed methods of artificial molt induction to occur at times other than at the time of natural molting. These methods have resulted in the cessation of lay and the loss of feathers. Historically, induced molting involved the removal of food; water or both feed and water. It also involved reducing the photoperiod to 10 h or less. Hens would then be fasted for a pre-set period of time to cause the complete regression of the reproductive tract (Berry, 2003). It was in the 1950's that egg producers in the United States first adapted the practice of induced molting. Egg producers at that time used multiple-molting programs in which hens were more than once and the laying cycles were shortened (37 weeks). The first molt was an average of

60 weeks and the second molt was induced at approximately 106 weeks. Californian producers were the first to adopt the practice (Bell, 2003) which spread across the United States to the most major egg producing regions in the mid 1970's. In 2000, the USDA stated that 93% of the flocks in the Southeast region of the US practice force molting intensively (USDA, 2000). Bell (2003) stated that approximately 73% of the commercial laying facilities in the US implement the practice of induced molting to rejuvenate their flocks. The practice is done to give the farmer maximum production from hens on the commercial layer farms by enabling them to have a second or even a third laying cycle. Under optimal economic conditions the useful reproductive life of laying hens can be extended from less than 80 weeks to more than 110 weeks or even 140 weeks (Bell, 2003; Webster, 1995) giving the producer second and third laying cycles. Lee (1982) stated that molting had beneficial effects on the individual hen's performance and the practice was also beneficial for the overall flock management since the hens were synchronized for the second laying cycle. If allowed to molt naturally, hens would begin molting at different times and this would prolong the process through the entire flock. Molted hens in this experiment had higher hen-day egg production than the non-molted control hens however; the molted hens consumed more feed post-molt than the control hens. Bell (2003) also observed improved post-molt performance in molted hens compared to their non-molted counterparts with peak of egg production during the second cycle of approximately 75% to 85% 13 weeks into the second cycle.

Naturally during the periods of short day length birds in the wild will experience weight loss along with feather loss and the regression of the reproductive system (Brake

and Thaxton, 1979; Mrosovsky and Sherry, 1980; Etches et al., 1996), during this time they will undergo a period of natural inappetence. In the commercial layer industry, an induced molt will usually mimic these conditions which will occur naturally. There are three basic ways by which a molt is induced; feed removal or limitation, low-nutrient ration, and feed additives. Each of these methods usually involves the alteration of the photoperiod from a long day to a short day. Whichever method is used should be easy to apply, low cost, result in low mortality and should result in enhanced performance during the second cycle (Swanson and Bell, 1975), the method should also avoid the potential feed refusal by the birds, be readily available, be economical with minimal feed processing, and provide a molt induction stimulus enough to cause sufficient reproductive tract regression during the molt (Ricke, 2003).

Feed Withdrawal

Commercial egg producers in the US typically use feed removal as their method to induce a molt. This because feed withdrawal is easy to implement and will achieve body weight loss of 25 to 35% to obtain the optimum post-molt performance in terms of egg production and shell quality (Webster, 2003; Baker et al., 1983). Feed withdrawal (deprivation) involves the removal of feed for a period of 5-14 days and the may or may not include a period of rest for up to 21 day after the fast (Bell and Kuney, 1992). Conventional feed withdrawal programs involves the removal of feed, water (no more than 3 days) or both from the hens and also reducing the photo-period in the houses to 8 h lighted period or less. Removal of feed will last for a period enough to cause the complete involution of the reproductive tract (Berry, 2003).

Feed withdrawal is considered harmful to laying hens (Webster, 2003) and presently no scientific literature exists on the effects of feed withdrawal on domestic hens. However, work has been done in other species of birds and the conclusion was drawn that these responses were similar within the avian species. Cherel et al. (1988) concluded that fasting (deprivation) could be categorized into three phases; phase 1 is usually short (few days) and involves a rapid decrease in body mass loss while at the same time there is a reduction in the rate at which the body mass is lost. Phase 2 is usually long term and may last several weeks or even months in some species. During this phase most of the energy used is from the catabolism of fat (Webster, 2003). Phase 3 can last from days to weeks and protein catabolism begins (Cherel et al., 1988). Protein catabolism will result in debilitation of the bird and eventually death. However, the degree of fasting that is imposed to induce a molt can be viewed as physiological adaptation of the hens (Webster, 2003). It has been reported that feed withdrawal alters the microenvironment of the crop and ceca in the intestines of the fowl (Impey and Mead, 1989; Durant et al., 1999; Ricke, 2003). Corrier et al. (1997) stated that though induced molting had no apparent effect on the pH or oxidation reduction potential of the ceca, it caused a reduction in acetic acid, propionic acid and total volatile fatty acids (VFA) in the ceca. Two years after Corrier's observations, Durant et al. (1999) observed increased crop pH, and reduced Lactobacilli populations and VFA concentrations in the crops of feed deprived hens.

Animal Welfare and Molting

Animal Welfare Issues

There have been increased concerns posed by animal welfare advocates about the effects of induced (forced) molting on hens (Webster, 2003). It is a common premise that systems that lead to injury, disease, deformity, or other physical signs of reduced health can mean that the welfare of the bird is compromised (Broom and Johnson, 1993; Fraser, 1995). There is a possibility that poor welfare can also result from confinement, food restriction or under-stimulation (Dawkins, 1999). Critics of forced molting have stated that the practice is cruel and in-humane since it involves the removal of food and this amount to starvation (Webster, 2000). Baxter (1994) reported that hens reared in caged systems experience chronic (long term) and acute (short term) suffering, as well as other threats to their welfare. This chronic suffering was observed on a continuous or repeated basis for the duration of the confinement in the cage and acute suffering was seen during the pre-laying period of the day. Anderson et al. (2004) postulated that antagonistic behavior such as aggression (observed between hens), escape and avoidance, and submission (used to determine fearlessness), and their relationship to stress within an animal could be potential indicators of welfare during molting.

Sherry et al. (1980) have shown that birds in the wild undergo a period of in-appetence followed by a natural molt and Webster (1995, 2000, 2003) stated that molting hens fared no worse than non-molted hens. Duncan and Petherick (1991) have argued that the welfare of the animal depends on how the animal feels and the claims that the animals' welfare is compromised only to the extent that the animal suffers.

There is however disagreement in how suffering can be assessed in birds. It is the common belief among welfare advocate groups that induced molting abuses the rules of the "Five Freedoms" for animals recommended by the United Kingdom's Farm Animal Welfare Council (Appleby et al., 2004). These laws are; freedom from hunger and thirst, freedom from discomfort, freedom from pain, injury and disease, freedom to express normal behavior and freedom from fear and distress. Duncan (1998) stated that symptoms of suffering were fear frustration and pain and these can be used to diagnose welfare. He further stated that increased aggression and stereotypic pacing were indicative of suffering and reduced welfare. Anderson et al. (2004) stated that the cage environment alters normal behavior patterns in laying hens and may increase the incidence of stereotyped behaviors that have been suggested to negatively influence the welfare of hens. Baxter (1994) concluded that the cages failed to provide for the laying hens' welfare needs since they were prevented from dust bathing, foraging, nesting and roosting. Efforts have been made to improve the cage environment for the hens by including enrichment facilities to improve the hens' welfare (Cooper and Albentosa, 2003). Webster (2003) have stated that induced molting does not adversely affect the behavior or the welfare of hens since they did not display high levels of displacement or abnormal behaviors such as stereotypy and increased aggression.

Animal Behavior

Cooper and Albentosa (2003) stated that generally behavioral repertoires of wild or free ranging animals in semi-natural environments may be used as crude indicators of the kinds of behaviors that might be deprived in captive conditions. When assessing a

birds' behavior as it relates to feed deprivation, it has been observed that hens that previously had access to a resource and are later prevented from free access, will exhibit a range of identifiable though non-specific behaviors that may be indicative of frustration (Cooper and Albentosa, 2003).

Molting by way of feed deprivation raise concerns of reduced animal welfare of the laying hen since it can be harmful and behavior might give direct indication of the well being of hens undergoing feed withdrawal (Webster, 2003, 2000). When examining the behavioral profile of domesticated hens, a number of specific parameters have being examined including, feeding, drinking, comfort, social, reproductive, and anti-predator behavior (Duncan, 1970). During molting there are several behavior patterns that are considered of particular interest and are potential welfare indicators of stress, these include submission- putting up no resistance, aggression- aggressive behavior towards neighbor, escape and avoidance- show of fear (Anderson et al., 2004), also observed are nonnutritive pecking- which is non-aggressive pecking at anything other than feed, preen- manipulation of the plumage with the beck, walk- locomotion involving one step or more, still- complete immobility of an alert hen, feeder- behavior directed into the feed trough and drink- ingestion of water from waterer (Webster, 2000). Some studies have looked at changes in aggressive behavior during induced molts. The results have been controversial. Aggrey et al. (1990) and Haskell et al. (2000) found that negative interactions among laying hens increased during feed deprivation in open housing systems but not in battery cages. Hembree et al. (1980) reported that the aggressive behavior of hens in colony cages during feed deprivation did not differ from hens not

deprived of feed. Webster (1995) found no significant differences in aggression between caged hens on a 4 day fast and hens that were not deprived of feed. When Anderson et al. (1989) investigated changes in the hens' environment; they observed that behavioral patterns changed significantly when hens were placed in cages. They concluded that the adaptation process lowered the number of negative social interactions between hens, which in turn reduced stress and enhance welfare. Tonic immobility was used by Jones (1989) to determine underlying states of fear. Zimmerman and Koene (1998) reported that calling and increased locomotion were indicators of frustration, in this case they identified a specific "gakel" call (Zimmerman et al., 2000). Other researchers observed increased aggression (Haskell et al., 2000), increased locomotion (Duncan, 1970; Duncan and Wood-Gush, 1971), increased pecking at feather bunches was observed by Haskell et al. (2000). While the prevention of feeding results in increased locomotor activity including stereotypic pacing, cage ground and feather pecking, aggression and gakel calling (Duncan, 1970; Duncan and Wood-Gush, 1971; Zimmerman and Koene, 1998), the intensity of these responses does not, however, correlate with the intensity of the deprivation. To date, only a limited number of studies have related deprivation of specific resources to behavioral or physiological measures of distress. Although there is no evidence that management programs involving feed withdrawal causes debilitation when properly implemented, uncertainty is still evident around the issue of whether or not the hunger involved in feed withdrawal causes suffering.

Immune Response during Molting

A variety of different physiological mechanisms contribute to the hens' reduced resistance during molting. Among the investigated mechanisms that cause decreased resistance are the alterations in the intestinal micro-flora (Corrier et al., 1997; Holt and Porter, 1992; Savage, 1989), intestinal physiology (Barnes et al., 1979; Corrier et al., 1997; Duke, 1986; Holt, 1993), and host defenses (Ben-Nathan et al., 1981, 1977; Holt, 1992a, b; Holt and Porter, 1992). During an induced molt, immunosuppressive effects on T-cell immunity (Holt, 1992a, b) and humoral immunity (Ben-Nathan et al., 1981, 1977) can be seen. Even with the massive invasion of inflammatory cells to the site of infection, molted hens are still more susceptible to infection. Inflammation is a sequence of complex reactions designed to attract effector cells to the site of infection to remove and destroy the inflammatory stimulants (Edwards, 1994). The suppression of the immune system during an induced molt is evident in the reduced antibody production, delayed type hypersensitivity, graft versus host response and increased circulating leukocytes with an increased H: L ratio.

Corticosterone

The hens response during an induced molt is somewhat related to the nutritional stress that is associated with the withdrawal of food. Dramatically elevated levels of corticosterone have been seen during acute feed restriction or fasting (Freeman et al., 1981; Harvey and Klandorf, 1983). In mammals, increased levels of corticosterone decreases inflammation thereby depressing the specific immune response. It does this by blocking interleukin-1 (IL-1) transcription (Besedovsky et al., 1986). IL-1 stimulates the

production of other cytokines including IL-2. These cytokines are responsible for activating the body's poly-morpho-nuclear (PMNs) cells such as macrophages that are the cells primarily responsible for inflammatory response. Corticosterone levels increase in hens as the hypothalamic-pituitary-adrenal (HPA) axis is activated by the body's need to mobilize energy. Juhn and Harris (1958) found that exogenous deoxy-corticosterone was as effective as progesterone in inducing molts. Etches et al. (1984) also reported elevated corticosterone levels in molting hens, they found that the levels to which corticosterone increased depended on the type of molt induced, for example, methods that induced a more rapid or complete molt, had a greater increase in the corticosterone levels. Stress causes a general deterioration of the well-being of animals and it usually involves a cascade of physiological adaptive responses (Thaxton and Puvadolpirod, 2000). Stress causes elevation of corticosterone levels in the blood and this result in increasing concentrations of glucose due to catabolism of muscle protein. The increasing levels of glucose due to gluconeogenisis results in increasing excretory uric acid which is an indication of protein degradation (Brown et al., 1958; Siegel and Van Kampen, 1984; Davison et al., 1985). Physiological stress responses observed in broilers as a result of adrenocorticotropin (ACTH) treatment includes elevation of plasma glucose (Siegel and Beane, 1961; Siegel, 1962), elevation of plasma cholesterol (Siegel and Siegel, 1966), and immuno-suppression of humoral and cell-mediated immune responses (Thaxton and Siegel, 1970; Holt, 1992a).

Circulating Leukocytes

Induced molting by dietary restriction can affect components of the birds' immune system. Brake et al. (1981, 1985) found that dietary restriction affected the thymus and spleen weights, resulting in the reduced production and maturation of T-cells. Other researchers found that the total number of circulating leukocytes decrease significantly during molting (Ben-Nathan et al., 1977; Brake et al., 1982; Holt 1992b; Holt and Porter, 1992) thereby reducing the hens' ability to fight infection. Wolford and Ringer (1962) previously found that molting influenced the differential white blood cell counts by causing a reduction in lymphocytes numbers and increasing heterophil numbers in the peripheral blood. Lane (1987) suggested that the avian heterophil could be used as the window to the state of their health and that the avian heterophil would respond to problems that are associated with their diet, chronic bacterial infections, stress light and trauma. In the majority of the avian species, lymphocytes are the most abundant leukocytes found in circulation (Maxwell and Robertson, 1998). In poultry, approximately 59% of the total white blood cells are lymphocytes and 27% are heterophil. In extreme conditions the heterophil/lymphocyte ratio (H: L) cannot always be used to provide an accurate assessment of stress, however, when heterophil behave consistently to a particular stressor, the H: L ratio can be used (Maxwell et al., 1992b). In light of this, H: L ratio has become widely accepted as a reliable and accurate physiological indicator of the stress response. Gross and Siegel (1993) have suggested that some reference value be used as guidelines to determine the different degrees of stress. H: L ratios of about 0.20, 0.50 and 0.80 are characteristic of low, optimal and

high degrees of stress, respectively. When Maxwell (1993) investigated H: L ratio and the role that corticosterone played when feed was withdrawn from hens, they observed an increase in the ratio. Jones (1989) found that fasted and frustrated Brown Leghorn pullets had an increased H: L ratio. However, when Maxwell et al. (1990) investigated broilers that were subjected to prolonged feed restriction, they found that the H: L ratios of these birds were not significantly altered when compared to birds that were fed *ad libitum.* These results confirmed results from studies that were previously done by Brake et al., (1982), Gross and Siegel (1989), Katanbaf et al., (1988) and Van Niekerk et al., (1988). This suggests that birds became habituated to the effects of prolonged feed restriction. During the early phase of feed restriction, heterophil populations responded to a hormonal surge (Maxwell et al., 1991, 1992b; Savory et al., 1992; Hocking et al., 1993, 1994, 1996), however, this increase cannot be physiologically sustained, therefore the heterophil will no longer be recruited to the site of infection. Alodan and Mashaly (1999) postulated significantly increased circulating leukocytes in molted hens over non-molted control hens. An increase in H: L ratio indicates that hens molted by way of feed restriction are under more stress (Alodan and Mashaly, 1999). They also elucidated that induced molting could result in inhibiting the immune response since the total circulating leukocytes in the early stages of the molting period was reduced when compared to control hens.

It should not be surprising to see the presence of heterophil at the sight of invasion because the avian heterophil is the primary phagocytic cell during an acute inflammatory

response (Powell, 1987). Kogut et al. (1998) elucidated that induced molting has a negative effect on the functional activities of the heterophil. They reported that while heterophil responded strongly to chemotactic stimulus, in molted hens the heterophil were unable to effectively phagocytize bacteria or illicit an oxidative burst to kill the *S.* Enteritidis. They also found a diminished phagocytosis of opsonized *S.* Enteritidis during an induced molt.

Recrudescence and lyphocytic repopulation usually accompanies reproductive quiescence in molting hens (Brake et al., 1981). Alodan and Mashaly (1999) found that peripheral lymphocyte decreased and lymphocyte population reduced during the fasting period of a molt. While Holt (1992b) found that there was a reduced number of B-cell observed in birds on a fasting induced molt, the antibody levels appeared to be reduced. Desmidt et al. (1998) stated that humoral immunity contributes to eliminate *S.* Enteritidis from the gut of poultry. Initially, *S.* Enteritidis occurs primarily occurs in the mucosal surfaces and a significant humoral immune response will generally occur in this area. Duchet-Suchaux et al. (1995) postulated that a humoral immune response is involved in the rapid elimination of *S.* Enteritidis from the ceca of some lines of chickens.

The immunoglobulin (Ig), IgA is the predominant immunoglobulin associated with mucosal immunity, however, IgG and IgM responses can also be observed. IgA can be found in both bile and mucosal secretions (Lebacq-Verheyden et al., 1972). In mammals, IgA serves as a possible opsonin for mucosal phagocytes (Kilian et al., 1988), protection against infection through antibody dependent cellular cytotoxicity (Tagliabue et al., 1983), inhibiting bacterial adherence (McGee et al., 1992), and neutralizing toxin moieties (Lange and Holmgren, 1978). Holt and Porter (1992) have observed substantial

mucosal immune response in experimentally challenged chickens against flagella and or lipopolysaccharides respectively in the intestines and bile. Seo et al., (2002) found high levels of IgA in response to *S.* Enteritidis infection in the crop (0.38 in infected hens and less than 0.10 in non-infected hens) and bile (1.17 in infected hens and less than 0.10 in non-infected hens). They also found that chickens infected with *S.* Enteritidis developed an antibody immune response as early as nine days in the serum and fourteen days in the egg. The degree of the antibody response is therefore correlated to the degree of infestation.

Serum Acute Phase Proteins

An acute phase response (APR) was the term coined to the phenomena observed in patients during the acute or early stages of an infectious illness (Fleck, 1989). The acute phase response has been described as the systemic, rather than local effects of inflammation and the term was first applied to the precipitin reaction observed when a pneumococcal extract was added to the serum from patients in the acute phase of pneumonia (Fleck, 1989). An APR is mediated by serum proteins, specifically acute phase protein (APP) is released into the bloodstream by a variety of stimuli including inflammation (Thomas and Schrieber, 1985; Fleck, 1989; Jamieson et al., 1992), bacterial infection (Morley and Kushner 1982; Pfeffer and Rogers, 1989) and endotoxin exposure (Takahashi et al., 1995). Researchers have found that the presence of inflammatory cytokines such as IL-1, IL-6 and tumor necrosis factor, enhances the synthesis of APP (Klasing, 1984; Marinkovic et al., 1989; Baumann and Gauldie 1990, 1994). Changes in the concentrations of APP in the serum, though nonspecific, can be a

sensitive means of indicating inflammation. Holt and Gast (2002) found that birds that were experimentally infected with *S.* Enteritidis and molted or non-molted, showed significantly higher APP levels than non-infected birds. Elevated concentrations of serum APP was also observed by Deignan et al. (2000) when they challenged calves with *S.* Enteritidis. The degree of increase was correlated to the severity of the disease. Salonen et al. (1996) and Godson et al. (1995) saw comparable effects in cows with mastitis and viral respiratory disease, respectively. Holt and Gast (2002) concluded that serum APPα_1 acid glyco protein (AGP) levels can be effective and rapid indicators of infection and can be useful in tracking the infection status of a flock. Xie et al. (2002) examined lipopolysaccaride (LPS) induced inflammation in chickens and found significant changes in not only blood concentrations of heterophil and IL-6, but also in serum protein profiles. They found that though the changes were seen in heterophil and IL-6, the serum proteins remained elevated up to 48h after treatment. In another study conducted by Xie and others (2002), they concluded that inflammation induced changes in several serum protein and that ovotransferrin is a positive APP in chickens. Although not specific, the determination of the levels of APP in the serum of molting hens can potentially be a quick and effective means of determining the infection status of a flock thereby stemming a potential outbreak of disease within the flock.

The Effects of Molting on *Salmonella enterica* serovar Enteritidis (*S.* Enteritidis) Infection in Laying Hens

In the mid-1980s, the international pandemic of *S.* Enteritidis began (Rodrique et al., 1990). *Salmonella* Enteritidis infections reached a high of 3.9 per 100,000 population

in 1985 and has since declined to 1.98 per 100,000 in 1999 (Patrick et al., 2004), however, while the total number of outbreaks declined by half, those in the western states tripled (Patrick et al., 2004). The spread of infection to humans is a consequence of the food poisoning form from chickens that are infected. The *S.* Enteritidis is spread not only from contaminated chicken carcasses, but also from eggs and the contents of intact eggs (Anonymous, 1989).

Feed withdrawal compromises the birds' immune system making them susceptible to infection by a number of microorganisms. Of particular interest is *S.* Enteritidis. Feed withdrawal associated stress causes increased susceptibility to *S.* Enteritidis infection (Holt, 1993; Corrier et al., 1997; Durant et al., 1999; Ricke, 2003; Holt, 2003) which is usually marked by increased intestinal shedding and colonization in internal organs such as the liver, spleen, and ovaries (Holt and Porter, 1992a; Holt, 1993; Holt et al., 1995; Thiagarajan et al., 1994). Other researchers have shown that emptying of the gastrointestinal tract by feed withdrawal is consistent with increased levels of *Salmonella* in a variety of animal species including chickens (Humphrey et al., 1993).

While feed withdrawal provides the benefits of extending the egg-laying life of the flock, it also has detrimental effects on the hens' immune system. In mammals and birds, deficient diets have been shown to reduce humoral immunity (Ben-Nathan et al., 1977, 1981; Gross and Newberne, 1980) and also cell-mediated immunity (Chandra, 1990; DePasquale-Jardieu and Fraker, 1979). Holt (1992a) observed that cell-mediated immunity was significantly depressed, while in another study (1992b), the B-cells and CD8+ T-cells were less infected. DePasquale-Jardieu and Fraker (1979) detected

elevated levels of serum corticosterone, which suggested that the hormone played a role in the depression of cell-mediated immunity. Brake and Thaxton (1979) and Etches et al. (1984) also noted a similar response when molting hens by way of feed withdrawal. The intestinal shed rate of *S.* Enteritidis is higher in birds that are exposed to exogenous sources of the pathogen concomitantly during molt induction. These hens had a more severe infection when compared to un-molted hens (Holt and Porter, 1992; Holt et al., 1995). These hens not only shed more organisms (Holt and Porter, 1992; Holt, 1993; Holt et al., 1994, 1995) but they also showed significantly more inflammation especially in the colon and cecum (Holt and Porter, 1992, Porter and Holt, 1993; Holt et al., 1995; Macri et al., 1997). Problems associated with *S.* Enteritidis in the flock environment can increase when birds are exposed to stresses such as feed deprivation and the spread of *S.* Enteritidis in larger numbers of susceptible hens in a flock is of major importance. Nakamura et al. (1994) observed that short-term feed removal can increase horizontal transmission to nearby hens, while Holt and Porter (1992) observed similar results when molting increased horizontal transmission to hens in neighboring cages. The impact of air-borne transmission is also a major concern since transmission to birds in cages which were a distance away from infected birds during a molt by feed withdrawal, was observed by Holt et al. (1998).

Laying hens with *S.* Enteritidis infected ovaries, will not only lay contaminated eggs, but infected chicks will also be hatched from these eggs. These chicks will grow to become pullets which will subsequently lay contaminated eggs (Hopper and Mawer, 1988; Lister, 1988; O'Brien, 1988). The process of molting can cause an increase in

infectivity of these birds (Cason et al., 1994) and stress situations such as feed withdrawal to induce a molt can also cause a recurrence of a previous *S.* Enteritidis infection (Hughes et al., 1989).

Holt et al. (1995) postulated that molting can significantly affect an *S.* Enteritidis infection at different times in the infection cycle when they observed that infected molted hens produced more eggs that were *S.* Enteritidis contaminated. They also observed that molting exacerbated the infection as shown by the ready transmission of the organism to previously uninfected hens, an indication that molting made these hens more susceptible (Holt et al., 1995). The extent to which feed withdrawal increases a hens' susceptibility to infection can be seen when comparing $10^{\wedge 3} - 10^{\wedge 4}$ colony forming units of *S.* Enteritidis necessary to infect 50% of a group of un-molted hens, while fewer than 10 organisms were necessary to infect molted hens (Holt, 1993; Holt et al., 1994).

Gast and Beard (1990) found S. Enteritidis in the albumin and yolks of eggs from hens 4 weeks after they were orally inoculated. This was an indication that internal contamination of eggs could occur prior to the laying of the egg, when *S*. Enteritidis was deposited on the shells. Keller et al. (1995) observed that when eggs which were not yet formed were removed from the oviduct they were contaminated with *S.* Enteritidis much more often than freshly laid eggs. Since induced molting by feed withdrawal is currently practiced by commercial layers, research into different methods by which to ameliorate the increased incidence of *S.* Enteritidis infection is necessary. The direction of interest to control this pathogen should be through intervention schemes or by using alternative methods for inducing a molt. Whichever products are used should be part of an

alternative method of inducing a molt and should be of economical advantage to the commercial farmers by successfully providing a second laying cycle of high production rates and good quality egg while at the same time reducing the incidence of increased susceptibility to pathogenic microorganisms.

Alternative Methods of Inducing a Molt

General Concepts

Due to the increased pressure and criticism of commercial layer farmers for their practice of feed deprivation as a means of inducing or forcing a molt, researchers have been investigating different methods to bring on a molt instead of depriving hens of feed. The goals of a successful molt are; for the hens to lose approximately 20 to 25% of their total body weight, for a cessation of lay long enough for the total regression of the reproductive tract and an acceptable and persistent second cycle performance (Scheideler et al., 2003). Generally speaking, egg production and quality deteriorate as the flock ages (Bell, 2003). After the rejuvenation of the reproductive tract the hens will come into a second cycle and produce better quality eggs (Keshavarz and Quimby, 2002). Bell (2003) observed that laying hens peaked in the first cycle in wk 8 at about 90% and in the second cycle in wk 13 at about 80%. Deterioration of the egg quality includes the internal and external traits of the egg and improvement in egg quality parameters are evident after an induced molt (Swanson and Bell, 1975). The parameters that are of particular importance include: albumin quality, candled grade, shell thickness, specific gravity, egg weight and shell texture (Bell, 2003).

Experimental molting techniques have varied widely in an effort to initiate a molt and cease egg production in a more humane way and also to alleviate problems of increased infectivity associated with feed withdrawal. Attempts have been made to restrict feed intake thereby reducing body weight and inducing a molt. This has been done by using nutritional imbalances, ingredients that decrease appetite, addition of fillers or administration of hormones to induce molts (Bell, 2003; Holt 2003; Park et al., 2004). Some of these methods include feeding a diet that contains less than 0.3% calcium (Douglas et al., 1972; Martin et al., 1973; Gilbert and Blair, 1975; Wakeling, 1978), feeding diets with less than 0.04% sodium (Whitehead and Shannon, 1974; Dillworth and Day, 1976; Nesbeth et al., 1976; Campos and Baiao, 1979; Ross and Herrick, 1981), feeding diets that contain high levels of zinc, usually in the form of zinc-oxide (Scott and Creger, 1977), and feeding diets containing iodine in the form of potassium iodine (Arrington et al., 1967). However, the results of some of these diets do not induce a consistent molt in all hens in the flock, most are either costly or managerially unwieldy and cannibalistic pecking has also been observed (Ricke, 2003; Park et al., 2004; Biggs et al., 2004; Webster, 2003).

High Fiber Low Energy Diets

A diet that is high in fiber is usually lower in metabolizable energy than a diet that has low fiber content (Wenk, 2001). Langhans (1999) stated that high fiber diets cause earlier satiety of an animal, while Rijnen et al. (1999) concluded that an animal that reaches satiety both physically and nutritionally is less stressed. With this in mind the incorporation of high fiber low energy diets to induce a molt has been suggested.

High fiber diets consist predominantly of plant cell walls, non-starch polysaccharides, and non-carbohydrate compounds including lignin, protein, fatty acids and wax (Bach Knudsen, 2001). By definition dietary fiber cannot be digested by endogenous processes, instead the resident microorganisms metabolize it (Wenk, 2001). These diets can alter the gastrointestinal tract by changing its microbial activities, the rate of passage, metabolites and the overall effectiveness of the tract (Bach Knudsen, 2001; Wenk, 2001). A variety of high fiber diets have been incorporated in poultry feed including cotton meal (Davis et al., 2002), jojoba meal (Arnouts et al., 1993), guar meal (McGinnis et al., 1983), grape pomace (Keshavarz and Quimby, 2002), wheat middlings (Biggs et al., 2004; Seo et al., 2001) and alfalfa (Landers et al., 2005 a,b; Woodward et al., 2005; Donalson et al., 2005).

Cotton seed meal is lower in energy and protein than soybean meal and can be used successfully incorporated in poultry diets. Davis et al. (2002) examined the effects of cotton seed meal when incorporated in layer diets. They observed that cotton seed meals at levels of 20 to 30% of the diet resulted in reduced egg sizes and yolk discoloration. Davis et al. (2002) reported no significant difference in egg production between the control hens and the hens fed feed containing cotton seed meal. There is however, disadvantages of using cotton seed meal in layer diet since it can result in the production of eggs with brown yolk discoloration.

Jojoba meal is a by-product after the extraction of oil from jojoba seeds. It contains approximately 30% crude protein which makes it potentially useful as an ingredient for animal feed. However, when Ngou Ngoupaya et al. (1982) fed jojoba meal

to chickens they observed impaired body weight gain, reduced feed intake and impaired feed efficiency. Arnouts et al. (1993) stated that broiler chickens fed a diet supplemented with 4% jojoba meal exhibited reduced growth rate. Vermaut et al. (1998) utilized jojoba meal to induce a molt in broiler breeder hens. These hens reached maximum body weight loss 5 wks after the start of the molt and stopped laying 3 wks after molt induction. They observed that the oviduct regressed to the same degree as the pair-fed hens. The oviduct re-grew completely after the jojoba meal was removed form these hens. This result was not observed by Vermaut et al. (1998) who fed jojoba meal to growing pullets and observed irreversible inhibition of oviduct development resulting in no egg laying.

Feeding guar meal diets to laying hens to induce a molt can be effective in causing greater loss in body fat and decreased loss in body protein when compared to a molt induced by feed withdrawal (McGinnis et al., 1983). Zimmermann et al. (1987) observed that laying hens stopped laying after being fed for 8 days with a 15% guar meal to a layer diet. They observed that these hens lost 31% of their body weight within 21 d.

When Keshavarz and Quimby (2002) molted hens using grape pomace, which is essentially the solid remains of grapes after the juice or oil has been pressed out. They observed that the hens that these hens stopped laying by days 3 to 4 after the initiation of the molt a similar time as the full feed withdrawal hens. They also noticed that the hens that were fed grape pomace lost a similar amount of body weight as the feed withdrawal hens (30.30 and 30.80% respectively). No significant differences were observed in egg production during the first 4 wk after the initiation of molt between the feed withdrawal

hens and the hens that were fed grape pomace (7.50 and 5% production respectively), also no differences were observed in specific gravity of the eggs or any other internal egg qualities. The reproductive organ weights (ovary and oviduct) of the hens treated with grape pomice were not significantly different from the hens which were deprived of feed. Although grape pomace can effectively induce a molt and have post-molt performance that is comparable to feed withdrawal hens, accessibility and storage issues could potentially be problematic due to its light low-density characteristic. Also based on the physiological response of hen on the grape pomice diet, it appears that this diet did not prove to be less stressful on the hens than the conventional method of feed withdrawal.

Wheat middlings are the by-products of wheat flour manufacturing. Wheat bran is also a by product of wheat manufacturing and contains higher fiber than wheat middlings which is utilized as a source of energy in animal feed (Bai et al., 1992). Biggs et al. (2004) evaluated the efficiency of wheat middling, corn gluten feed and distillers dried grains with solubles (DDGS) as alternative methods of inducing a molt. Previously, Biggs et al. (2003) showed that diets high in wheat middling or corn, were effective methods that obtained post-molt performances that were comparable to a 10 day feed withdrawal molt. In a latter study, Biggs et al. (2004) observed that all diets (wheat middling, corn gluten feed, and distillers dried grains with soluble) yielded decreased egg production and body weight during the molting period. However, they found that the DDGS diet had the highest oviduct and ovary weight (2.50% and 3.50% respectively of their body weight) which suggest that there was limited ovarian

regression using DDGS. Biggs et al. (2004) postulated that feeding hens with diets high in corn, wheat middling, corn gluten feed or 71% wheat middling 23% corn combination could be used as effective alternative molt programs. They observed no significant differences were observed in post-molt egg parameters among treatments. When Seo et al. (2001) examined wheat middlings as a dietary source during a molt they observed that the hens which were fed wheat middlings stopped laying within 7 d after the beginning of the molt. Biggs et al. (2004) observed that while the feed deprived hens in their study ceased egg production within 6 d, the hens that were in the other treatments did not totally stop producing eggs even though the hens in the wheat middlings treatments reduced production at a faster rate the hen in the other treatments. Post-molt feed intake and feed efficiency of hens fed wheat middlings during a molt is not different from that of hens which were molted by the conventional feed withdrawal. The acceptance of the feed initially by the hens was poor and hens fed wheat middling ate two fold less feed than other treatments.

Potential of Alfalfa as a Molting Diet

Alfalfa as a Dietary Ingredient for Poultry

As early as the mid 1920's scientists have shown that alfalfa leaf, alfalfa meal, and alfalfa hay can be a source of vitamin A in poultry diets (Beach, 1924; Davis and Beach, 1925; and Kennard and Lingle, 1928). Heywang (1932) stated that alfalfa leaf meal can be a satisfactory source of vitamin A when fed at levels of 7.50 % to 10% of the total feed intake. Alfalfa is a forage crop that is easily accessible in most and poultry producing regions of the United States and is currently commercially available in mash,

pelleted and cube form. It contains 17.50% crude protein and 24.10% crude fiber and has low levels of metabolizable energy, approximately 1200 kcal/kg when compared to layer ration which has a metabolizable energy of 2,965 kcal/kg (NRC, 1994). Dehydrated alfalfa is one of several feedstuff that spends more than 24 h in the chickens' digestive tract (Sibald, 1979) and the longer the retention time in the ceca and the intestines, the greater the opportunity for microbial degradation of fiber and the production of volatile fatty acids (Hungate, 1966). Alfalfa leaf meal when fed in sufficient amounts (5% -10% of the diet) to furnish 0.2 mg of carotene per bird daily prevented the occurrence of deficiency lesions in the throats of birds (Williams et al., 1938). Heywang (1950) also observed that when White Leghorn laying hens were fed diets containing 5% to 25% sun-cured alfalfa meal had a reduction in total egg production occurred as the level of the alfalfa in the diet increased. Draper (1948) observed a significant decline in weight when chicks were fed alfalfa meal and the rate of weight reduction increased as the quantity of alfalfa in the diet increased from 5% to 15%. Cooney et al. (1948) reported that there was an unidentified factor in alfalfa that resulted in a reduced growth of New Hampshire chicks when fed at 10% of the diet. Germen and Couch (1950) also observed a depressed growth in New Hampshire chicks and the depression in growth was proportional to the levels of alfalfa in the diet. Heiman and Wilhelm (1937) observed that yolk pigmentation was greater for hens which were fed alfalfa than those hens which were fed corn; this is a desirable trait for consumers in some regions. Kohler (1972) has also stated that alfalfa was an excellent source of pigment for broiler and laying hens. Sen et al.(1998) and Ponte et al.(2004), found that alfalfa was well balanced

in amino acids, rich in vitamins, carotinoids and xanthophylls that give poultry carcass its' desirable yellow color. It also contained high levels of bioactive antinutritive factors such as saponins (Sen et al., 1998). Saponins are considered steroids or triterpenoid glycosides that possess hypocholesterolemic, anti-carcinogenic, anti-inflammatory and antioxidant properties (Rao and Gurfinkel, 2000; Ponte et al., 2004). Williams and Bolin (1938) concluded that when alfalfa was fed in sufficient quantities to laying hens to furnish 0.2 mg of carotene per bird daily, alfalfa also kept the birds in good health and resulted in fair egg production. Ponte et al. (2004) found that meat from broiler hens fed moderate levels of alfalfa was acceptable to consumers.

Fermentation products of the microorganisms present in the gastrointestinal tract can limit foodborne pathogen colonization. McHan and Shotts (1993) observed a 50-80% reduction in *Salmonella* Typhimurium population in the presence of short chain fatty acids. Dietary fiber are preferentially utilized by *Lactobacillus* and *Bifidobacteria* species which leads to the production of lactic acid and short chain fatty acid (Kaplan and Hutkins, 2000), this results in a low pH which will maintain the normal microorganism population thus preventing the establishment of *Salmonella* in the gastrointestinal tract (Juven et al., 1991). Donalson et al. (2004a,b) observed that alfalfa can enhance the fermentation properties of cecal microflora which are capable of limiting *in vitro* growth of *Salmonella* Typhimurium. Sibbald, (1979, 1980) observed of the slow passage rate of alfalfa through gastrointestinal tract of the chicken. This could be potentially beneficial as it allows for better digestion of the feed and microbial fermentation thereby maintaining a balanced microflora which will limit pathogenic

mircorganisms in the gastrointestinal tract (Ricke et al., 1982; Vispo and Karasov, 1997).

Alfalfa Molt Diets and Egg Production

The utilization of alfalfa in laying hens nutrition has been attempted to improve yolk pigmentation and also as a protein concentrate (Burdick and Fletcher, 1984; Miller et al., 1972). The potential of alfalfa as a molt diet is evident due to its' slow passage and low energy content leaving the birds with a feeling of satiety (Sibald, 1979, 1980). When high levels of alfalfa were incorporated in the diets of laying hens, Whitehead et al. (1981) observed that the saponins contained in the alfalfa depressed feed consumption, body weight, egg production liver lipid concentration and plasma triglyceride concentrations.

Donalson et al. (2005) incorporated alfalfa as a molt diet and reported that the hens ceased production of eggs by day 5 of the molt as opposed to day 4 by the feed withdrawal hens. Hens fed alfalfa diets obtained weight loss that was comparable to that of the feed withdrawal hens (Woodward et al., 2005; Donalson et al., 2005). No significant differences were observed between the feed deprived hens and the alfalfa fed hens when examining the length of time it took for the birds to return to 50% to 60% production. Donalson et al. (2005) observed that hens fed 100% alfalfa ration returned to lay after an average of 14.8 days after molt induction, with the feed withdrawal hens achieving up to 74.29% egg production by 39 weeks post-molt. This was not significantly different from the hens that were fed 90% alfalfa diets, similar levels of egg production post-molt were also observed by Landers et al. (2005a). Landers et al.

(2005a) and Donalson et al. (2005) observed that hens which were molted using alfalfa diets produced eggs with interior qualities that were comparable to the eggs produced by the feed withdrawal hens. They also observed that hens which were fed 100% alfalfa diets consumed the least amount of feed and lost weight that was comparable to feed withdrawal hens. Donalson et al. (2005) also reported that hens fed 90-100% alfalfa had second cycles that were comparable to the feed withdrawal hens they observed that hens which were fed 70% alfalfa lost the least amount of weight and took the longest amount of time to cease egg production. Landers et al. (2005b) conducted a sensory study that evaluated the consumer assessment of eggs produced by the hens which were treated using alfalfa diets. The sensory investigation was based on the taste, texture and color of the cooked egg. They found that the eggs produced by hens molted with alfalfa, were not considered less desirable than those produced by hens molted by feed withdrawal. Donalson et al. (2005) evaluated the changes in the internal organs of the hens fed alfalfa molt diets compared to those hens which were molted by feed withdrawal, they observed no significant differences in ovary or oviduct weight among the hens in the molting treatments (alfalfa or feed withdrawal), indicating that the reproductive tract of the hens on the diet were regressed. Landers et al. (2005 a,b) found similarities when they compared ovary regression weights between hens molted by feed withdrawal and hens molted using alfalfa.

Alfalfa Molt Diets and S. Enteritidis Infection

When used to induce a molt in laying hens, alfalfa limited *S.* Enteritidis colonization and infection during the molt (Woodward et al., 2005), they observed no

significant difference between the alfalfa diets and full fed hens in cecal enrichment positives. Woodward et al. (2005) observed no differences between the treatments in *S.* Enteritidis colonization of the crop and postulated that the presence of lactate within the crop could effectively inhibit *Salmonella.* Alfalfa was efficacious in reducing *S.* Enteritidis colonization in the spleen, liver and ovaries (Woodward et al., 2005) and can also increase volatile fatty acids such as acetate, butyrate and propionate. McReynolds et al. (2005) combined alfalfa with an experimental chlorate product and observed results similar to that of non-molted birds. They observed an increase in lactic acid production, reductions in organ colonization and also, cecal content colony forming units (cfu). When comparing the effects of high fiber diets on *S.* Enteritidis infection in molting hens, alfalfa diets, alfalfa with chlorate products (Woodward et al., 2005; McReynolds et al., 2005, 2006) and wheat middlings (Seo et al., 2001) favorably reduced the levels of *S.* Enteritidis in the ceca, ovaries, livers and spleen. The use of wheat middlings also resulted in reduced numbers of *S.* Enteritidis in the feces. Hens fed alfalfa diets had higher concentrations of volatile fatty acids than feed withdrawal hens.

CHAPTER III

BLOOD PLASMA METABOLITE RESPONSE IN MOLTING LAYING HENS CHALLENGED WITH *SALMONELLA* ENTERITIDIS AND FED ALFALFA CRUMBLE DIETS

Introduction

Molting in domestic hens is a naturally occurring experience, however, it is usually incomplete and hens will continue to lay eggs at low rates for an extended periods resulting in un-profitability due to a reduction of egg production and egg quality (Bell, 2003). To the commercial egg producers this is an indication of the end of the useful life in a flock and at the first sign of molting commercial egg producers would either sell these hens and replace them with a new flock or induce a molt. Egg producers usually make the decision to molt hens for a second laying cycle based on the cost of eggs and the cost of feed and replacement pullets (North and Bell, 1990). The cost of replacement pullets is one of the most expensive costs for a layer operation; therefore, commercial egg producers seek to extend the productive laying life of their flock for as long as it is profitable (Parkhurst and Mountney, 1988). Whatever method of inducing molt that is used usually involves the changing of the lighting period. Commercial egg producers who adapt the practice in the US typically use feed removal as their method of inducing a molt. Feed is removed for a period of 5-14 days and the program may or may not include a period of rest for up to 21 day after the fast to bring birds back into maximal production (Bell and Kuney, 1992). The practice of feed withdrawal has been described as stressful (Beving and Vonder, 1978) and stress causes a general

deterioration of the well-being of animals and it usually involves a cascade of physiological adaptive responses (Thaxton and Puvadolpirod, 2000). Puvadolpirod and Thaxton (2000a,b,c,d) have studied physiological stress in chickens extensively and stated that it can be determined by the evaluation of blood metabolites. Weber et al. (1990) reported that feed withdrawal was stressful to chicks since it resulted in elevated levels of plasma corticosterone.

Researchers over the years have developed various methods to induce a molt such as nutritional imbalances or the incorporation of high fiber low energy diets to induce a molt (Webster, 2003; Park et al., 2004). A variety of high fiber diets have been incorporated in poultry feed including cotton meal (Davis et al., 2002), jojoba meal (Arnouts et al., 1993), guar meal (McGinnis, 1983), grape pomace (Keshavarz and Quimby, 2002) and wheat middlings (Biggs et al., 2004; Seo et al., 2001). Alfalfa has the potential to serve as dietary source for inducing a molt. When used to induce a molt in laying hens, alfalfa meal limited *Salmonella* Enteritidis (SE) colonization and infection during the molt (Woodward et al., 2005; McReynolds et al., 2005, 2006). Landers et al. (2005a,b) found that hens molted with alfalfa meal or alfalfa pellets had ovary regression weights that were equivalent to that of hens molted by feed withdrawal and retained acceptable egg production characteristics in the second laying cycle. Donalson et al. (2005) observed similar results when they examined molting layer hens fed with different ratios of alfalfa meal and layer ration.

One problem associated with the use of alfalfa meal as a molting diet has been reduced feed intake and wastage of the feed (Woodward et al., 2005). Landers et al.

(2005b) examined alfalfa meal and alfalfa pellets and reported that hens fed alfalfa pellets reentered egg production earlier than hens fed alfalfa meal. Woodward et al. (2005) concluded that the reduced intake observed in hens fed alfalfa meal could account for the incomplete elimination of SE from the ceca of infected hens. It has been shown that using feeding pellets or crumbles improved growth rate and feed conversion in chickens (Heywang and Morgan, 1944; Kilburn and Edwards, 2001). Allred et al. (1957) observed similar results whether the feed was pelleted or if it was reground. They concluded that this was indicative of a change in the nutritive value of the feed due to the pelleting process. Nir et al. (1994) explained that the superior performance observed with pelleted or crumbled food is because they are more suitable for the chickens' digestive system than a mash diet with uniform particle size. The development of an alternate diet to feed withdrawal is essential to alleviate concerns of reduced animal welfare and increased infectivity. Our over-all goal is to examine alfalfa crumble diet as an alternative molt induction diet and compare hens' physiological response to the physiological stress that usually accompanies feed deprivation. Mumma et al. (2006) stated that endocrine and biochemical changes are definitive responses to stress in most species, including adult fowls. Elevated plasma levels of corticosterone, glucose, cholesterol, total protein and triglycerides are associated with stress in chickens (Siegel, 1995; Puvadolpirod and Thaxton, 2000). In this study we determined the effects of alfalfa crumble molt diets on blood plasma metabolites.

Materials and Methods

Experimental Design

Experiments 1 and 2 were conducted using 250 Single Comb White Leghorn hens (SCWL) over 50 weeks old, which were obtained from a local commercial laying flock. Twelve hens were used in each of 6 treatments in trial 1 and 10 hens were used in each of 6 treatments in trial 2. The hens were placed in wire layer cages and were given free access to water and un-medicated corn-soybean meal based mashed layer ration that met National the Research Council recommendations for nutrients (1994). Based on published data the alfalfa crumble diet was considered to be high in crude fiber (24.1%), with moderate crude protein (17.5%) and low in metabolizable energy (1200 kcal/kg; NRC, 1994). Feed and fecal samples (1 g) were collected and examined for salmonellae. Samples were cultured in tetrathioninate broth and on brilliant green agar (BGA) plates as was previously described by Andrews et al. (1995). All the hens and feed used in both trials tested negative for *Salmonella.*

The hens were allowed to acclimatize in the cages for a period of two weeks after which 12 (10 in the case of trial 2) hens were randomly assigned to 6 treatment groups designated as followed; (1) feed withdrawal SE+ (FW+), (2) full fed SE + (FF+), (3) 100% alfalfa crumble SE + (ALC+), (4) feed withdrawal SE - (FW-), (5) full fed SE -, and (6) 100% alfalfa crumble SE - (ALC-). The molt procedure used to induce the molt was a modification by Holt (1993) which was previously described by Brake et al. (1982). Treatment diets were applied to each treatment group on d 1 of the molt, at the

same time feed was removed from the feed withdrawal hens. Treatment diets were administered for 12 days, the period of time the FW hens were deprived of feed. Hens in all the treatment groups were given water *ad libitum*. The SE positive hens were all placed in the same room, while the SE negative hens were placed in a separate room. Foot baths were placed at the doors of all the rooms in the facility and non-infected hens were cared for before the infected hens each day. The hens in all the treatment groups were exposed to 8 h light: 16 h dark photo-period one week before changing the diets and removing feed from the FW hens. This light schedule continued for a 12 day period after which the experiment was terminated.

Bacterial Strain

For this experiment a primary poultry isolate of *S.* Enteritidis (phage type 13A) from the National Veterinary Services Laboratory, Ames, Iowa, selected for resistance to novobiocin and nalidixic acid (NO and NA) in the USDA-ARS facility, College Station, TX, was used. The media used to culture the resistant isolate contained 25 µg of NO and 20 µg of NA per ml. The culture was prepared from an overnight culture previously transferred three times in trypticase soy broth. The challenge inoculum was prepared by serially diluting the culture in sterile phosphate buffered saline (PBS) to a concentration of approximately 10^6 cfu per ml. The cfu of the challenge inoculum was confirmed by plating on BGA plates. On d 4 of the molt, all the hens in groups 1, 2 and 3 were challenged by crop gavage with 1ml of inocula containing approximately 10^6 cfu of NA and NO resistant SE. The challenge dosage was slighter higher than the $5.6x10^4$ cfu dosages reported by Holt (1993) to be the mean

infectious dosage for *S*E in molted hens. Groups, 4, 5 and 6 were not challenged with SE.

Blood Collection and Chemical Analysis

Twenty-one gauge, 1.5 inch needles were used to collect approximately 8 ml blood from the jugular vein in the neck of 12 birds which were randomly selected on at the beginning of the study to obtain the baseline data. Eight hens were bled in a similar manner from each treatment group on days 2, 5, 9 and 12 of the molt. Eight ml was placed in 10 ml non-heparinized blood collection tubes. The plasma was separated by centrifugation at 2500 rpm X 15 min, the clear supernatant plasma from each sample were collected and placed in plastic vials and were stored at -20° C until chemical analysis could be conducted.

A clinical chemistry analyzer (Gilford Impact, 400E, Ciba Corning Dianostic Corp., Oberlin, OH 44774) was used to analyze spectrophotometrically, the concentrations of the plasma metabolite parameters in each of the samples using methods as described by Kubena et al. (1985) and Park et al. (1999). Chemical reagents obtained from Bayer HealthCare (Bayer Diagnostics, Europe Limited, Chapel Lane, Swords, Co. Dublin, Ireland) were used as outlined in the manufacturer's manual to determine the concentration of the following plasma metabolites: calcium, cholesterol, total protein, glucose, triglyceride, and uric acid. Cuvettes were loaded with 1 ml of the respective reagent and 20 µl of the sample. The cuvette were agitated to ensure proper mixing of the liquids and then allowed to incubate at room temperature for approximately 5 min, after which they were read by the chemical analyzer.

Statistical Analysis

The concentrations of blood plasma metabolites were summarized in each treatment and means of each metabolite over the 12 d period were analyzed using a repeated measures design. SAS Proc GLM (SAS version 8.3, SAS Institute Inc., Cary NC, 2001) was used with treatment, time, and treatment by time interaction and individual hens nested within treatment as the factors. Chicken nested within treatment was the error term used to test for treatment effects. When significant ($P \leq 0.05$) treatment by time interactions was found, means were compared using Least Significant Difference.

Results and Discussion

Feed Intake and Weight Loss

The feed intake response to the alfalfa crumble diet in both experimental trials exhibited significant differences between the FF groups and the ALC groups by as much as 6 fold in both trials (Table 3.1). Over the 12 day molt period for both trials the FW+ (34.82 and 35% in trial 1 and 2 respectively) hens lost significantly more ($P \leq 0.05$) body weight than the FF+ (1.67 and 1.50% in trials 1 and 2 respectively) and the ALC+ (18.40 and 20% in trials 1 and 2 respectively) hens lost significantly more weight than the FF+ hens.

Table 3.1: Effects of alfalfa crumble diet on feed intake and weight loss in laying hens experimentally infected with *Salmonella* Enteritidis (SE) trials 1 and 2

Treatment	Trial 1		Trial 2	
	Feed intake g/hen/day	%Weight loss /hen	Feed intake g/hen/day	%Weight loss /hen
FW+	N/A	34.82 ± 2.47^{a}	N/A	35.00 ± 3.00^{a}
FF+	90.19 ± 3.48^{a}	1.67 ± 2.43^{d}	86.78 ± 4.43^{a}	1.50 ± 1.30^{d}
ALC+	16.17 ± 3.97^{b}	18.40 ± 3.44^{c}	17.56 ± 3.42^{b}	20.00 ± 2.10^{c}
FW-	N/A	29.00 ± 3.21^{ab}	N/A	32.00 ± 2.10^{ab}
FF-	73.22 ± 8.78^{a}	1.14 ± 4.58^{d}	82.75 ± 3.68^{a}	-1.00 ± 1.90^{d}
ALC-	15.07 ± 6.09^{b}	20.45 ± 3.47^{bc}	18.43 ± 5.67^{b}	26.00 ± 2.70^{bc}

[a-d] Means within columns with no common superscript differ significantly ($p<0.05$). FW+= Feed withdrawal SE positive hens, FF+= Full fed SE positive hens, ALC+= Alfalfa crumble diet SE positive hens. FW-= Feed withdrawal SE negative hens, FF-= Full fed SE negative hens, ALC- =Alfalfa crumble diet SE negative hens.

This was consistent with results observed by Woodward et al. (2005) and Landers et al. (2005b) when they demonstrated that alfalfa fed hens lost approximately 19% of their total body weight when compared to FF hens which actually gained weight. Donalson et al. (2005) reported in a molting study that feed deprived hens lost up to 25.80% of their total body weight which did not differ significantly from hens fed a diet with 100% alfalfa meal (25.10%) or a diet with 90% alfalfa meal. The weight loss could be a result of the regression of the reproductive system (Donalson et al., 2005). Baker et al. (1983) demonstrated that approximately 30% reduction in body weight is needed for the ovary to successfully regress. Berry and Brake (1985) stated that approximately 25% of the body mass loss can be attributed to reduction in liver and reproductive organ weights. Brake (1993) later concluded that ovarian weight loss occurred simultaneously with body mass loss. Donalson et al. (2005) and Landers et al. (2005a) observed no significant difference between feed withdrawal hens and alfalfa molted hens in ovary weight. The body weight loss of these hens was significantly lower than the non-molted hens which also exhibited significantly higher ovarian weight. Other explanations for the weight loss seen in the ALC hens could be the reduced feed and water intake, low metabolizable energy in alfalfa, and also these hens could be using their fat reserves and they ate much less than the FF hen and therefore could not maintain their body weight.

The difference in weight loss between the hens in both ALC groups and the hens in both FW could be a result of the slow passage of alfalfa through the gastrointestinal tract of the ALC hens making the intestines of the tract heavier than the FW hens. Mrosovsky and Sherry (1980) reported that birds had suppressed appetite during a

natural molt, and Sen et al. (1988) concluded that reduced feed intake by hens fed alfalfa could be as a result of the low palatability. Matsushima (1972) reported that the saponins present in alfalfa could play a role in the suppression of feed intake and growth. Earlier, Sibbald (1979) demonstrated the slow passage rate of alfalfa (more than 24 h) through the chickens' gastrointestinal tract he concluded that this gave the birds a feeling of satiety causing them to reduce their intake.

Calcium Concentration

Significant treatment by day effect was observed for calcium concentration (Figure 3.1) in both trials 1 and 2 ($P \leq 0.001$). On d 2 of both trials no significant differences ($P \geq 0.05$) were observed between the ALC+ hens and the other treatment groups in calcium concentration. On d 5 in trial 1 the FF- hens exhibited significantly higher ($P \leq 0.05$) concentrations of calcium in the blood than the other treatment groups and in trial 2 the FF+ groups had higher levels ($P \leq 0.05$) of calcium than the ALC+ hens and FW+ hens which were not significantly different ($P \geq 0.05$). A similar trend was observed on d 9 in both trials when the FF+ hens exhibited higher concentrations of calcium than the FW+ and ALC+ hens which were not significantly ($P \geq 0.05$) different in trial 1. On d 12 in trial 1 the FF+ hens exhibited significantly higher ($P \leq 0.05$) levels of calcium than the FF- and FW- hens but not the other treatments. The ALC+ hens did not display significant differences ($P \geq 0.05$) from the other groups. The on the final day of the molt in trial 2 the concentrations of calcium were significantly higher ($P \leq 0.05$) in the FF+ hens than the FW+ and ALC+ hens.

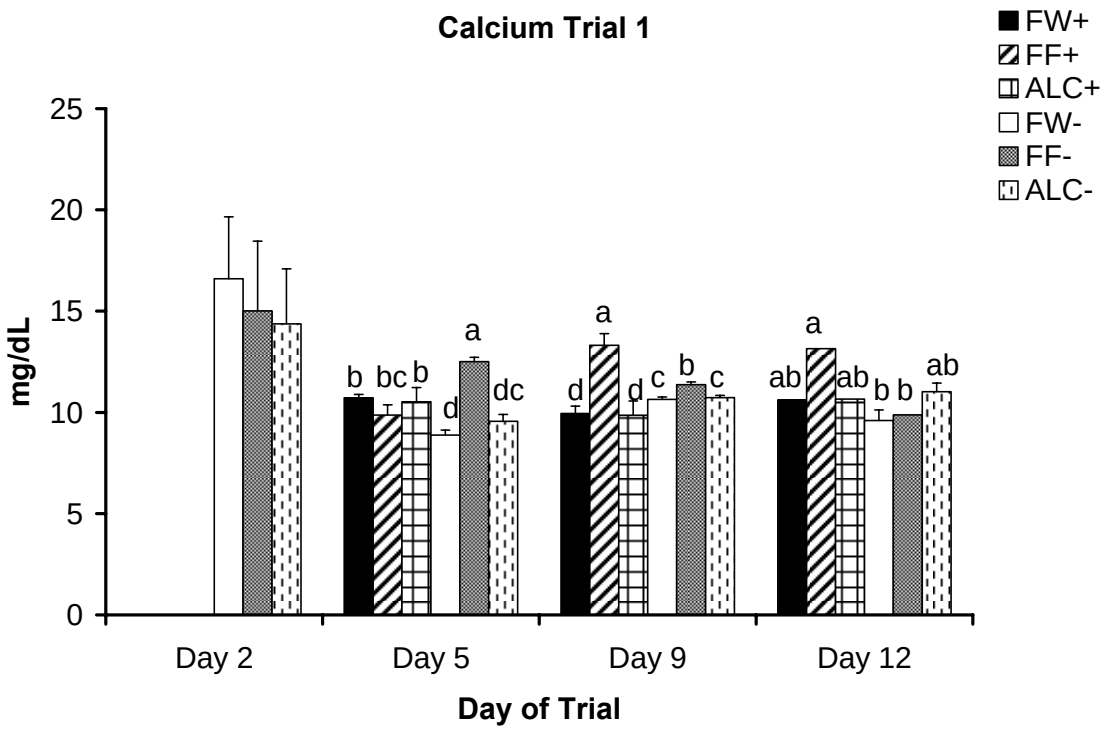


Figure 3.1: Effects of alfalfa on blood calcium levels in molting laying hens on days 2, 5, 9, and 12 after molt initiation in trial 1 and trial 2. Hens were infected on d 4 of the trial therefore there were no SE positive hens on d 2.
FW+= Feed withdrawal SE positive hens, FF+= Full fed SE positive hens, ALC+= Alfalfa crumble diet SE positive hens. FW-= Feed withdrawal SE negative hens, FF-= Full fed SE negative hens, ALC- =Alfalfa crumble diet SE negative hens.
Treatments within individual days that have no common superscript are significantly different ($p \leq 0.05$).

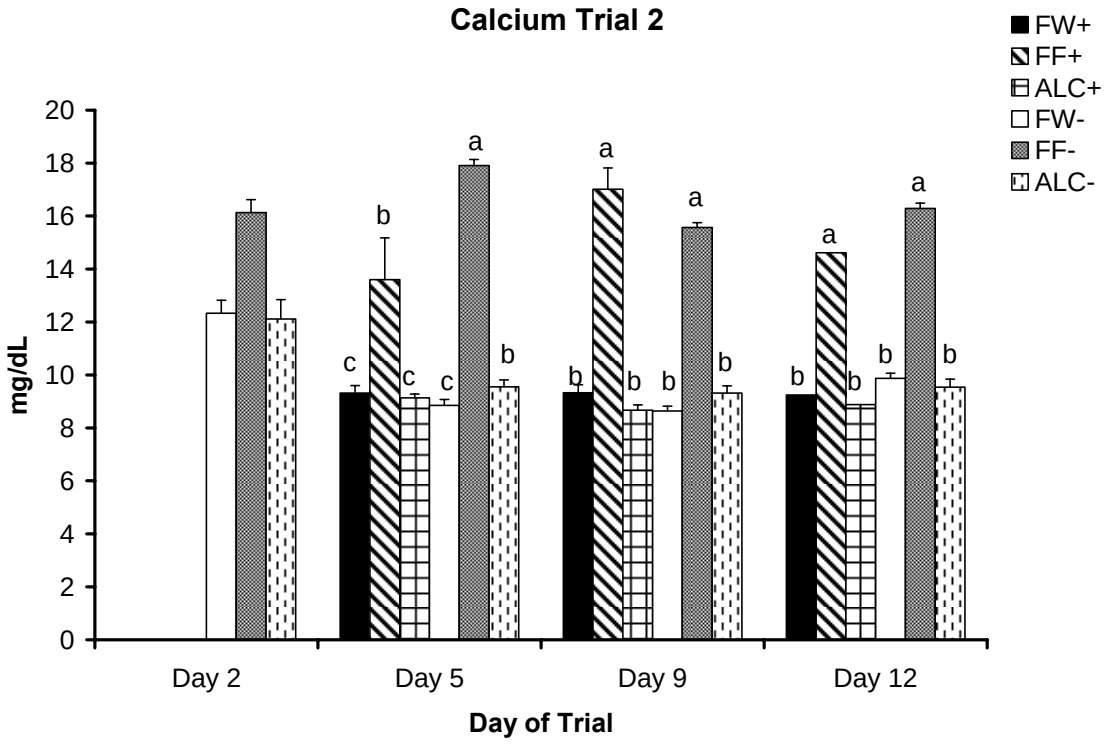


Figure 3.1: Continued

Laying hens require high levels of calcium in the diet to sustain egg shell production this was evident in both trials where we observed the FF hens had higher levels of the metabolites in the blood. Calcium is mobilized from the bones and intestines and transported to the reproductive tract for deposition in the shell gland; therefore it would be expected that the FF hens have higher levels of calcium in the serum the FW hens and the ALC hens because they had ceased egg production. Yosefi et al. (2003) observed decreased levels of egg shell gland and the intestinal calbindin during molting due to its dependency on calcium. Calbandin is present in the intestines of birds before the onset of reproduction to accommodate high calcium demands for shell calcification (Striem and Bar, 1991).

Total Protein Concentration

A significant treatment by day effect was observed in total protein concentration (Figure 3.2) in the plasma ($P \leq 0.001$)in both trials 1 and 2. There were no significant differences ($P \leq 0.05$) in total protein concentration among the treatment groups on d 2 in trial 1 however; in trial 2 the FF+ hens exhibited significantly higher concentrations of total protein than the ALC+ and FW+ hens on d 2. On d 5 in both trials the SE+ groups exhibited no significant differences ($P \geq 0.05$) in total protein concentration, while on d 9 in both trials, the FF+ hens exhibited higher ($P \leq 0.05$) concentrations of total protein (8352.50 and 4666.67 mg/dL in trial 1 and 2 respectively) than the ALC+ hens (5950 and 3783.33 mg/dL in trial 1 and 2 respectively) and the FW+ hens (6662 and 3641.67 in trial 1 and 2 respectively). The ALC+ and FW+ hens did not differ significantly. This

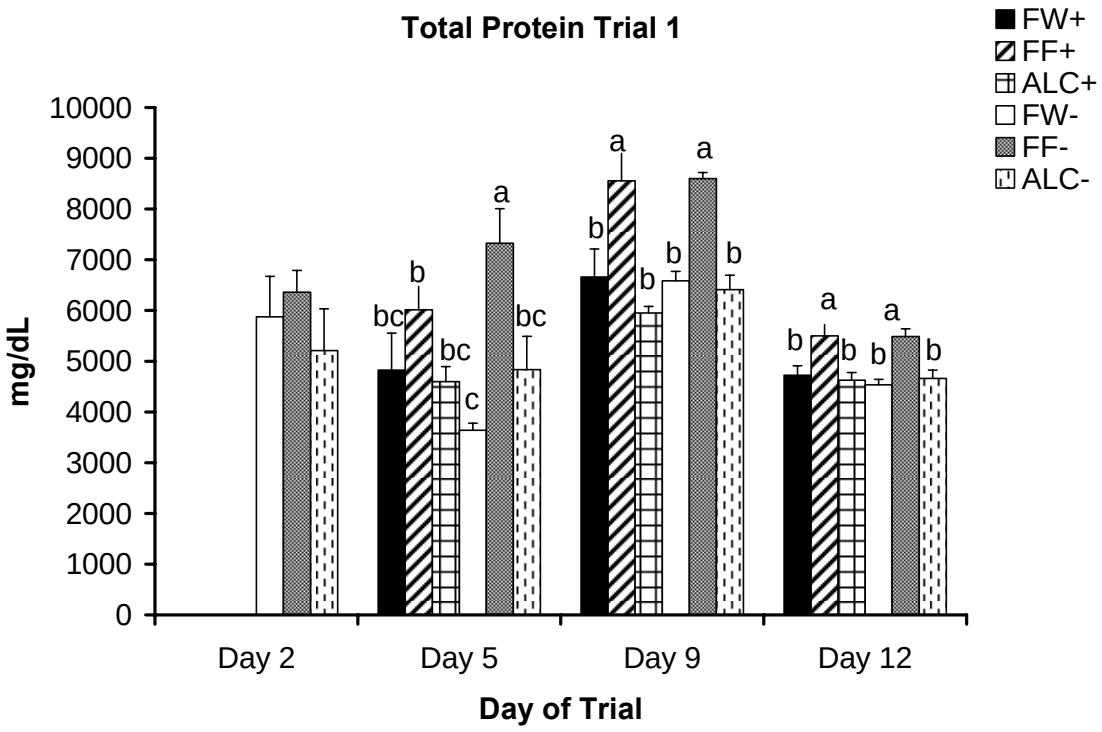


Figure 3.2: Effects of alfalfa on blood total protein levels in molting laying hens on days 2, 5, 9, and 12 after molt initiation in trial 1 and trial 2. Hens were infected on d 4 of the trial therefore there were no SE positive hens on d 2.
FW+= Feed withdrawal SE positive hens, FF+= Full fed SE positive hens, ALC+= Alfalfa crumble diet SE positive hens. FW-= Feed withdrawal SE negative hens, FF-= Full fed SE negative hens, ALC- =Alfalfa crumble diet SE negative hens.
Treatments within individual days that have no common superscript are significantly different ($p \leq 0.05$).

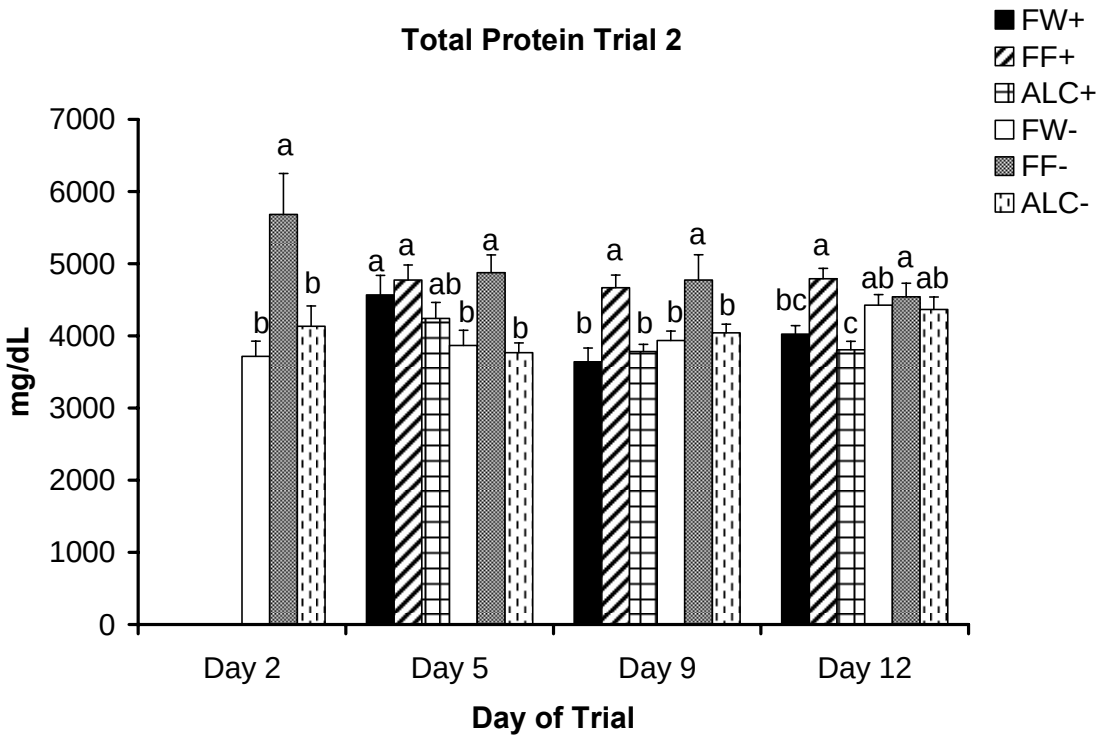


Figure 3.2: Continued

result was repeated on d 12 of the trial 1when we observed that the FF+ hens again exhibited higher ($P \leq 0.05$) levels of total protein than the hens in the other treatments. In trial 2 on d 12 the FF+ hens exhibited higher ($P \leq 0.05$) levels of total protein than the FW+ hens and the ALC+ hens. The negative treatments did not differ significantly ($P \geq 0.05$) from FF+ and the FW+ hens but they differed ($P \leq 0.05$) from the ALC+ hens.

The amount of protein required for integumental synthesis such as feather synthesis is less than one-third of that deposited in a single egg (Murphy, 1994), however, molting involves an acceleration of the total protein turnover (Murphy and Tarusico, 1995) and protein lost during an induced molt is also being restored (Harms, 1983; Hoyle and Garlish, 1987). Puvadolpirod and Thaxton (2000a) reported elevated levels of total protein in ACTH treated chickens (3960 mg/dL) when compared to non-treated chickens (3410 mg/dL).

Triglyceride Concentration

Significant treatment by day effects ($P \leq 0.0002$) were observed in the levels of triglycerides (Figure 3.3) found in the blood plasma in trials 1 and 2 during the nine day trial. On d 2 in both trials no significant differences were observed among the treatments. On d 5 in trial 1 all FF hens exhibited significantly higher ($P \leq 0.05$) levels of triglycerides in the blood than all FW and ALC hens. Similar results were observed in trial 2 on d 5 but the FF- hens exhibited higher ($P \leq 0.05$) levels than all the other groups (3990 mg/dL) and the FF+ hens exhibited higher ($P \leq 0.05$) levels (2665 mg/dL) than all FW hens and all ALC hens. Significant differences ($P \geq 0.05$) were not observed among any of the ALC

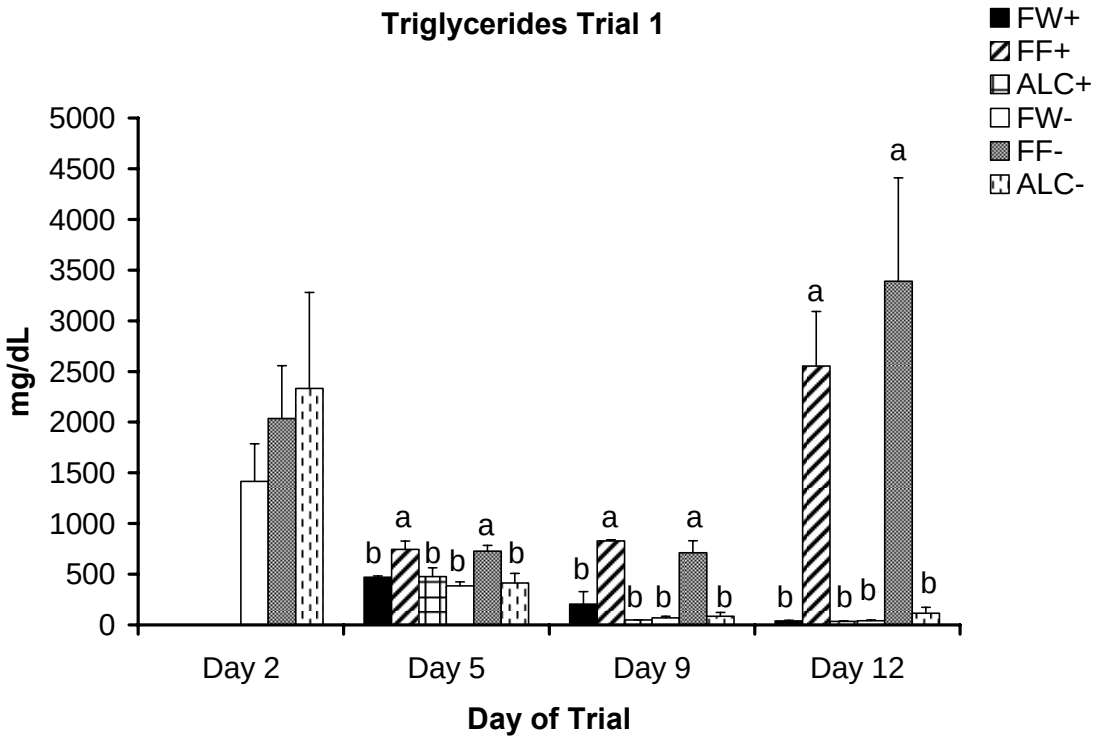


Figure 3.3: Effects of alfalfa on blood triglycerides levels in molting laying hens on days 2, 5, 9, and 12 after molt initiation in trial 1 and trial 2. Hens were infected on d 4 of the trial therefore there were no SE positive hens on d 2.
FW+= Feed withdrawal SE positive hens, FF+= Full fed SE positive hens, ALC+= Alfalfa crumble diet SE positive hens. FW-= Feed withdrawal SE negative hens, FF-= Full fed SE negative hens, ALC- =Alfalfa crumble diet SE negative hens.
Treatments within individual days that have no common superscript are significantly different ($p \leq 0.05$).

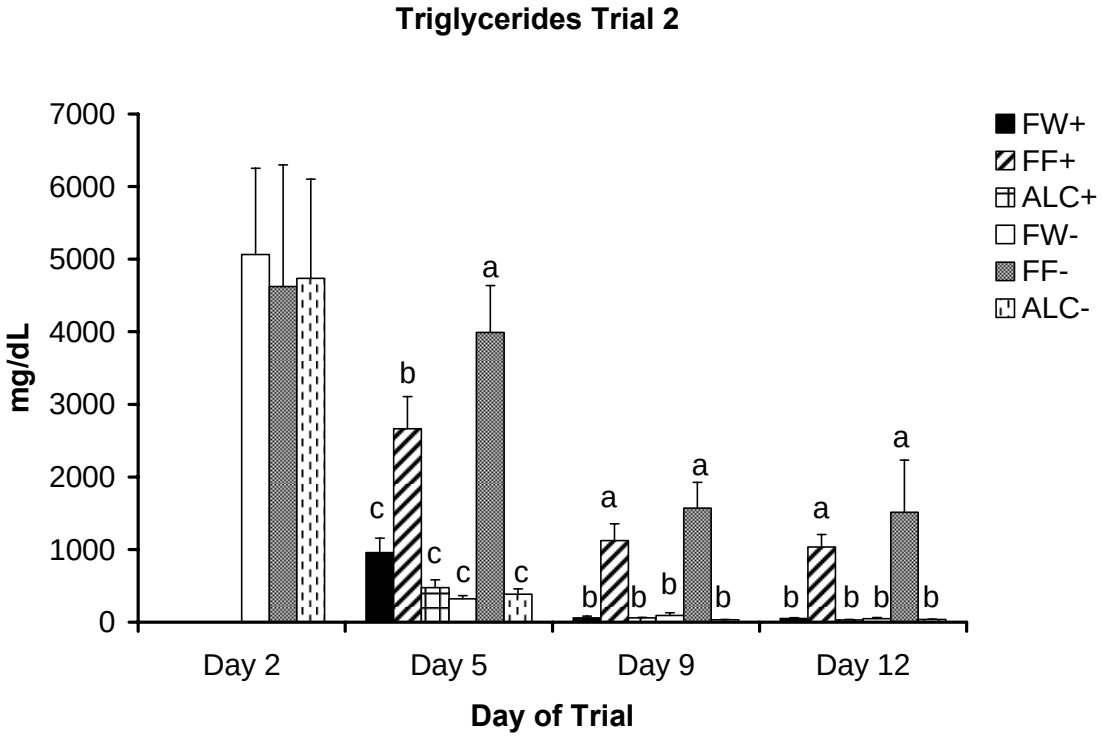


Figure 3.3: Continued

or FW treatments. The trend of reducing levels of triglycerides in the blood of the FW hens and the ALC hens was observed on d 9 in both SE+ and SE- hens in both trials.All FF hens exhibited significantly higher ($P \leq 0.05$) levels of triglycerides than all ALC hens and all FW hens. All the ALC and FW hens exhibited similar ($P \geq 0.05$) levels of triglycerides in the blood on d 9. The lowest concentrations of triglycerides were observed on d 12 in both trial 1 and 2.

In general the SE+ and SE- hens in the FW and ALC treatments in both trials, exhibited significantly lower levels of triglycerides than the FF hens. High levels of triglycerides are in circulation in laying hens, mobilized from the liver for the purpose of follicular development, during reproductive rest, the amount of triglycerides in circulation is reduced. This could be related to the reduced ovarian weight observed by Landers et al. (2005a) when they molted hens with alfalfa meal (5.1 g), pellet (7.0 g) feed withdrawal (6.2 g) and compared the results to non-molted hens (35.5 g). Puvadolpirod and Thaxton (2000a-d) stated that an elevation in triglyceride levels could be used as a stress indicator, this elevation in triglycerides results from the transportation of the triglycerides from the liver to tissues such as the muscles and adipose tissues (Assmann and Jerzy-Roch, 2003). Apparently this was not the case in the hens which were molted in the current study since these hens did not display increased levels of triglycerides in the blood. Anthony et al. (1999) also observed reduced plasma concentrations of triglycerides in fasted hens which were challenged with *Pasteurella multocida* and Mumma et al. (2006) observed decreased plasma levels of triglycerides in

laying hens during adreno- corticotrophin hormone induced stress while they observed an increase in broilers.

Cholesterol Concentration

There was no treatment by day effects ($P > 0.85$) in cholesterol concentration (Figure 3.4) in both trials 1 and 2, we however observed treatment effects. No significant differences ($P > 0.05$) were observed between the ALC+ hens and the other treatment groups in levels of cholesterol in the plasma on d 2 of both trials and 2 however; in trial 2 the FF+ hens had significantly higher levels of blood plasma cholesterol than the FW+ hens. Significantly elevated ($P \leq 0.05$) cholesterol concentrations were observed in the FW+ hens on d 5 in both trials 1 and 2. On d 5 the ALC+ hens exhibited levels similar ($P > 0.05$) to the FF+ hens in trial 1 and 2. No significant differences ($P > 0.05$) were observed in trial 1 on d 9 and 12 among the treatment groups in cholesterol concentration In trial 2 on d 9 the FW hens had significantly higher ($P \leq 0.05$) levels of cholesterol than the FF+ hens, the ALC+ hens were not significantly different from the FF+ hens. On d 12 in trial 2 the FW- hens had significantly higher levels of cholesterol than all ALC hens.

Throughout the molt the FW and ALC hens displayed increasing levels of serum cholesterol. This increase was not always significantly different at $P \leq 0.05$ from the FF hens which at times exhibited lower levels of cholesterol in the plasma. Walzem et al. (1994) postulated that this trend was expected in molted hens, as the resorption of atretic follicles during the process of molt should increase the amount of circulating

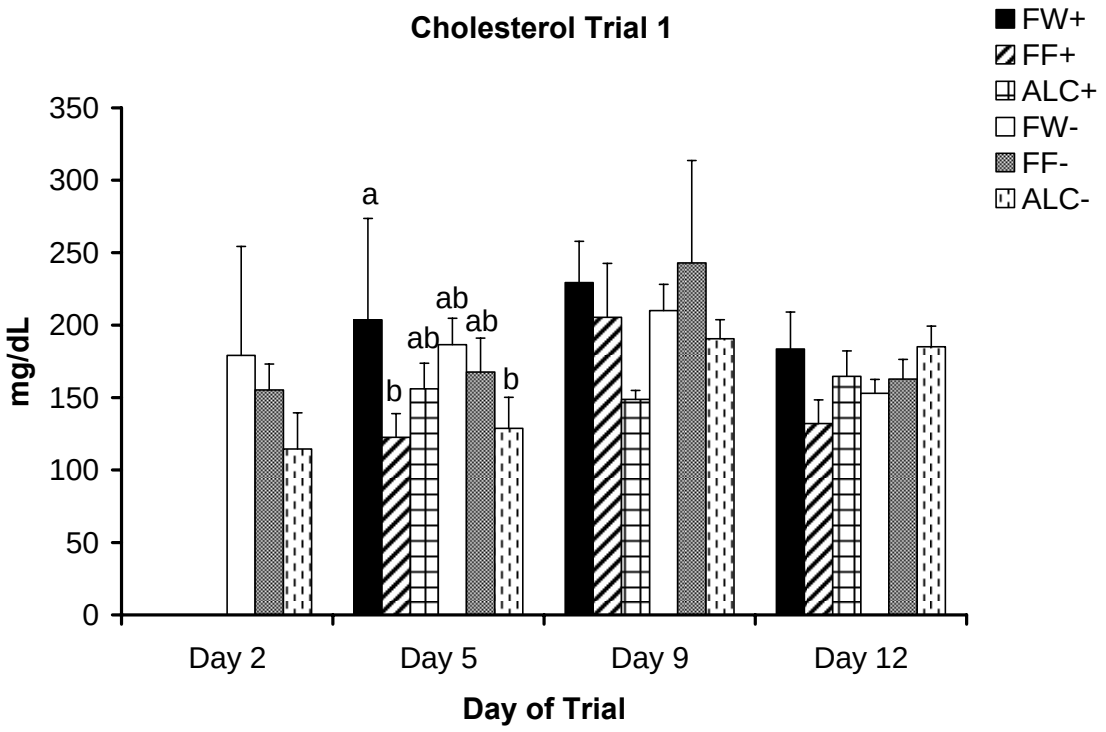


Figure 3.4: Effects of alfalfa on blood cholesterol levels in molting laying hens on days 2, 5, 9, and 12 after molt initiation in trial 1 and trial 2. Hens were infected on d 4 of the trial therefore there were no SE positive hens on d 2.
FW+= Feed withdrawal SE positive hens, FF+= Full fed SE positive hens, ALC+= Alfalfa crumble diet SE positive hens. FW-= Feed withdrawal SE negative hens, FF-= Full fed SE negative hens, ALC- =Alfalfa crumble diet SE negative hens.
Treatments within individual days that have no common superscript are significantly different ($p \leq 0.05$).

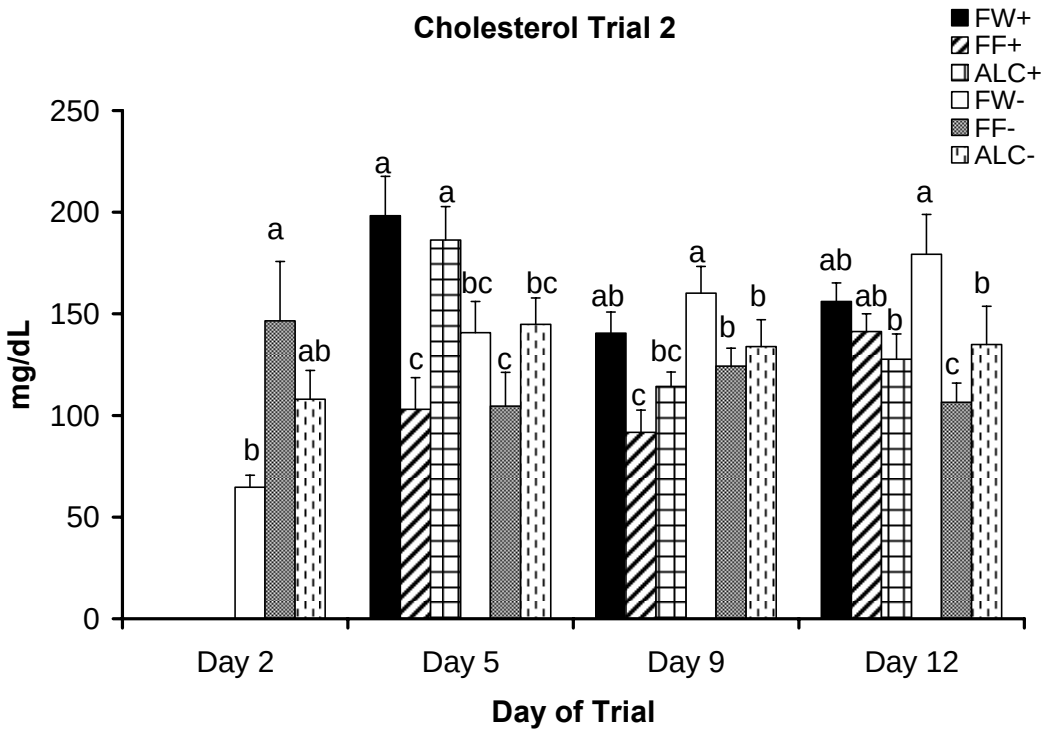


Figure 3.4: Continued

cholesterol in the blood, also, failure to ovulate could cause an increase in insoluble fraction of cholesterol, resulting in higher concentrations of cholesterol.

Mumma et al. (2006) stated that plasma cholesterol levels are increased during stress and Siegel (1995) stated that elevated levels of plasma cholesterol were associated with stress in chickens. Puvadolpirod and Thaxton (2000) stated that stressed birds exhibited elevated levels of plasma cholesterol. The reduced levels of cholesterol observed in the ALC hens in this study could be a result of alfalfa saponins which can reduce the levels of serum cholesterol in the blood. It is said that alfalfa saponins form insoluble complexes with cholesterol in the gut lumen (Coulson and Evans, 1960). This is consistent with the reduction in blood plasma cholesterol when saponins were fed to chicks and adult roosters observed by Griminger and Fisher (1958).

Glucose Concentration

No treatment by day effect ($P > 0.2$) was observed in glucose concentration (Figure 3.5) in the plasma however, there were treatment effects in both trial 1 and 2. In trial 1 all the FF hens exhibited higher levels ($P \leq 0.05$) of glucose in the blood than the ALC+ hens, the FW+ hens was not significantly different ($P > 0.05$) from the FF+ hens or the ALC+ hens. On d 2 in trial 1 the ALC+ hens did not display significantly different levels of glucose than the FW+ hen or the FF+ hens, however, the FF+ hens exhibited higher levels of blood glucose than the FW hens. On d 5 of the molt in trial 1 the FF+ hens had significantly higher ($P \leq 0.05$) glucose concentrations when compared to the FW+ and the ALC+ hens which were not significantly different. In trial 2 on d 5, the FF+ hens were not significantly different ($P > 0.05$) from any of the other treatment

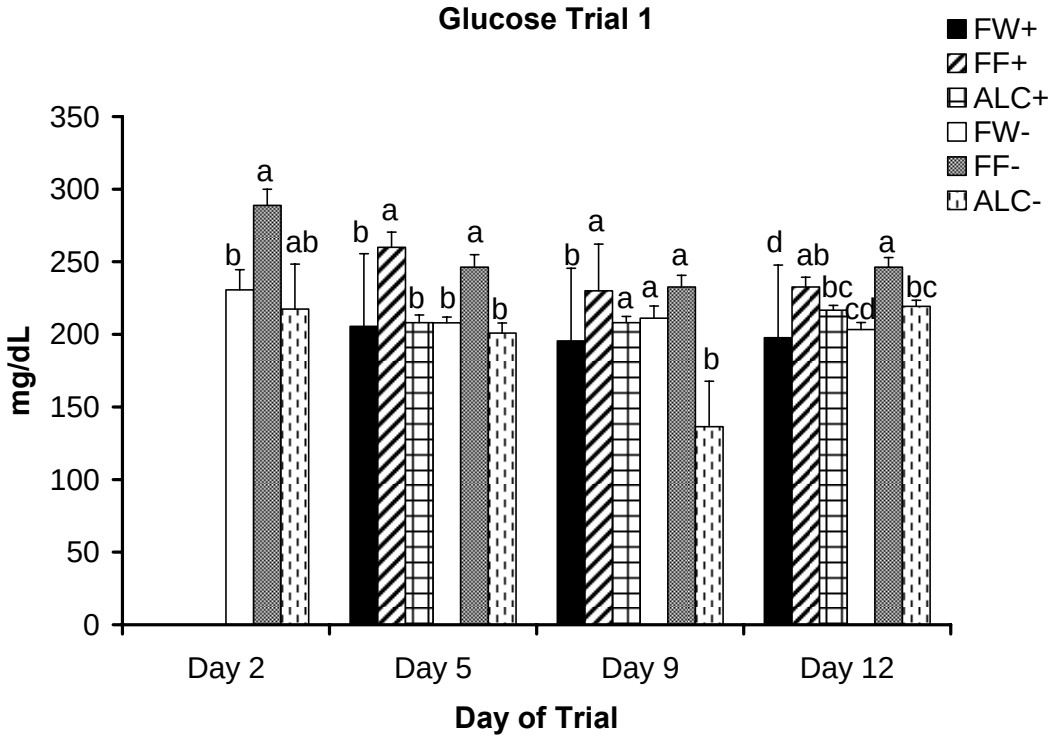


Figure 3.5: Effects of alfalfa on blood glucose levels in molting laying hens on days 2, 5, 9, and 12 after molt initiation trial 1 and trial 2. Hens were infected on d 4 of the trial therefore there were no SE positive hens on d 2.
FW+= Feed withdrawal SE positive hens, FF+= Full fed SE positive hens, ALC+= Alfalfa crumble diet SE positive hens. FW-= Feed withdrawal SE negative hens, FF-= Full fed SE negative hens, ALC- =Alfalfa crumble diet SE negative hens.
Treatments within individual days that have no common superscript are significantly different ($p \leq 0.05$).

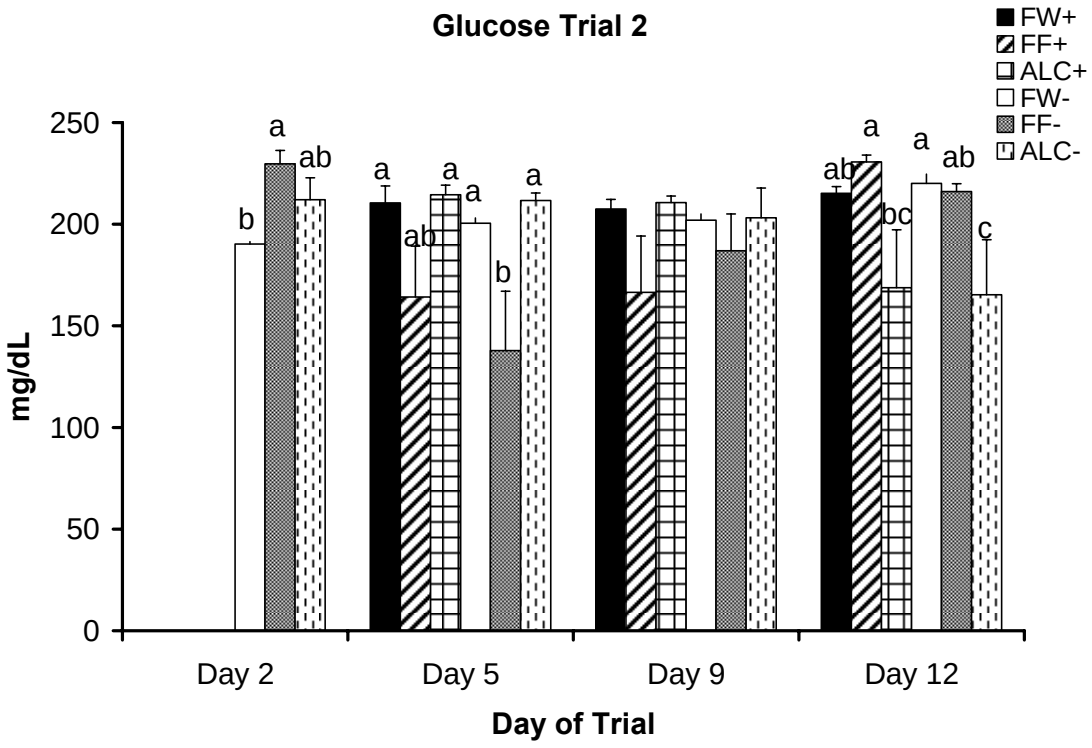


Figure 3.5: Continued

groups, the ALC+ and FW+ hens were not significantly different ($P > 0.05$). In trial 2 on d 5, the FF+ hens were not significantly different ($P > 0.05$) from any of the other treatment groups, the ALC+ and FW+ hens were not significantly different ($P > 0.05$). The results of glucose concentration on d 9 of the trial 1 showed the FW+ and the ALC- hens exhibited the lowest ($P \leq 0.05$) concentrations of glucose and there were no differences between the other treatment groups. No differences ($P > 0.05$) were observed among the treatments in trial 2. On d 12 of trial 1 the FW+ hens exhibited lower ($P \leq 0.05$) levels of glucose in the blood than the FF+ hens and the ALC+ hens. This was not the same in trial 2, the ALC- hens exhibited the lowest levels of plasma glucose and was significantly different($P \leq 0.05$) from the other treatment groups except for the ALC+ hens.

The FW+ and FW- hens were deprived of feed for a total of 12 d while the ALC+ and ALC- hens were given a low calorie alfalfa diet yet both the FW hens and the ALC hens exhibited glucose levels that were at times similar to the FF+ and FF- hens. Plasma glucose concentrations in chickens will generally decline at the beginning of feed restriction (Katanbaf et al., 1989; Edwards et al., 1999) however, Scanes et al. (1980) did not observe reductions in glucose concentrations in feed restricted adult birds. Anthony et al. (1999) also observed depressed glucose concentrations during feed deprivation. Puvadolpirod and Thaxton (2000a) reported that glucose levels increased in ACTH stressed induced chickens. Cherel et al. (1988) investigated molting fasts in king penguins and observed that glucose did not change substantially during molting. Nagra

and Meyer (1963) stated that increased circulating glucocorticoids induce gluconeogenesis which leads to elevation in plasma glucose.

Uric Acid Concentration

Significant treatment by day effect ($P < 0.0001$) was observed in uric acid concentration (Figure 3.6). On d 2 uric acid concentration of the FW+ (2.04 and 2.03 mg/dL for trials 1 and 2 respectively) and the ALC+ (1.75 and 2.25 for trials 1 and 2 respectively) hens were significantly reduced ($P \leq 0.05$) when compared with the FF+ hens (5.14 and 5.83 in trials 1 and 2 respectively). On d 5 of the molt in trial 1 the FF+ hens had significantly higher ($P \leq 0.05$) uric acid (4.90) concentration when compared to the FW+ (1.86) and the ALC+ hens (2.61), which did not significantly differ. The FF+ group had higher levels of uric acid (6.15) than the ALC+ hens (3.12) and FW hens (2.67) groups in trial 2. The ALC+ and FW+ hens did not differ significantly. On d 9 of the molt the FW+ hens exhibited lower concentrations of uric acid (3.32 in both trial 1 and 2) than the FF+ hens (5.95 and 5.72 for trials 1 and 2 respectively) and the ALC+ hens (5.05 and 5.72 for trials 1 and 2 respectively), the ALC+ and FF+ did not differ significantly. On the final day of trial 1, the ALC hens exhibited higher levels of uric acid than the FW hens. No significant differences were observed among the treatments on d 12 in trial 2.

At the beginning of the trial a reduction in the uric acid concentration was observed in both the FW+ and FW- hens and the ALC+ and ALC- hens. For the FW hens this could have been the result of the lack of a protein source which the hens would usually get from the diet. The ALC hens initially did not readily accept the alfalfa diet at

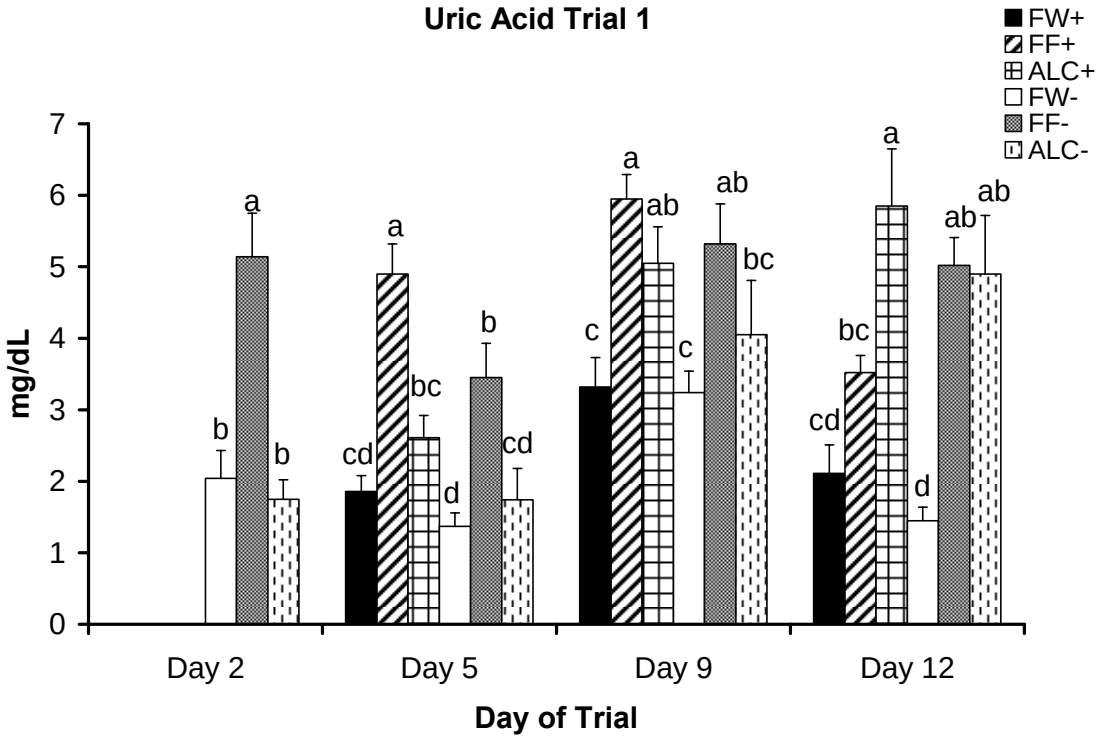


Figure 3.6: Effects of alfalfa on blood uric acid levels in molting laying hens on days 2, 5, 9, and 12 after molt initiation in trial 1 and trial 2. Hens were infected on d 4 of the trial therefore there were no SE positive hens on d 2.
FW+= Feed withdrawal SE positive hens, FF+= Full fed SE positive hens, ALC+= Alfalfa crumble diet SE positive hens. FW-= Feed withdrawal SE negative hens, FF-= Full fed SE negative hens, ALC- =Alfalfa crumble diet SE negative hens.
Treatments within individual days that have no common superscript are significantly different ($p \leq 0.05$).

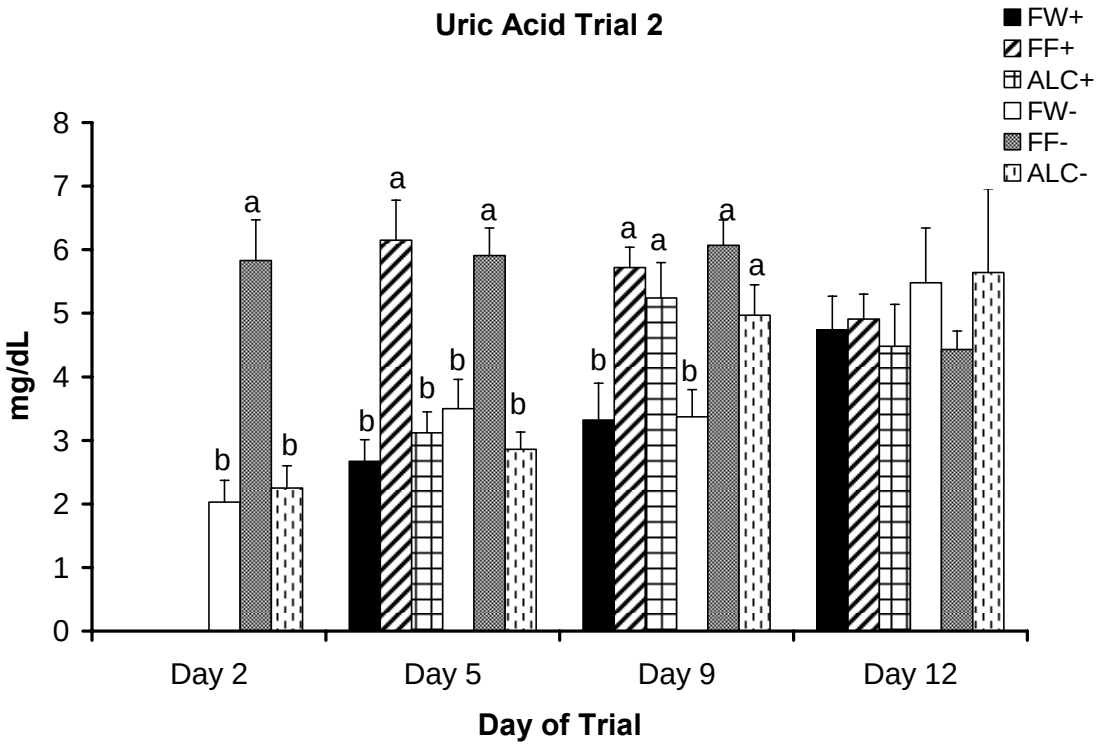


Figure 3.6: Continued

first as a feed source which could account for the reduction in uric acid concentration observed at the beginning of the trial. Puvadolpirod and Thaxton (2000a) observed increased levels of uric acid in stressed birds. Cherel et al. (1988) found that plasma concentration of uric acid was highly variable, increasing after the thirteenth day of the fast, which was an indication of the utilization of endogenous proteins. Anthony et al. (1999) observed low uric acid concentration early after feed deprivation in slow growing turkeys and reported that the rate of protein degradation increased with the duration of feed withdrawal and protein degradation was indicated by plasma uric acid levels. The levels of uric acid in the molting groups steadily increased towards the end of the molt period, the ALC+ hens exhibited higher levels of uric acid than the FW+ hens, which could be a result of increased microbial activity within the gastrointestinal tract of the ALC hens when compared to the FW hens.

Thaxton and Puvadolpirod (2000) advised caution when attempting to diagnose stress using physiological changes since it consisted of a series of non-specific adoptive response that facilitates the return to homeostasis, however, blood plasma metabolites can be used as indicators of increased stress, based on work done by other researchers. In general, the non-challenged hens exhibited levels of blood plasma metabolites that were similar to their SE challenged counterpart, therefore the presence of an infective organism in the hens did not alter the results even though this would mean the SE+ hens were exposed to an additional stressor. It has been established that induced stress results in the elevation of the blood plasma metabolites triglycerides, glucose, cholesterol, total protein and uric acid. These elevations were generally not observed in the alfalfa fed

hens as they displayed reducing levels of triglycerides which could be associated with the regression of the reproductive tract. The alfalfa fed hens displayed glucose levels that were comparable with the full-fed hens, while the SE+ FW hens exhibited means glucose levels for the molt period that was higher than the fed hens, whether, alfalfa fed or full-fed. The total protein levels exhibited by the ALC hens were at times lower or similar to the FF hens for the trial and could be a result of reduced feed intake during the period. At the beginning of the trial the ALC hens exhibited reducing levels of uric acid, this however changed and by the end of the trial these levels were similar to that of the FF hens. This can be interpreted as the result of protein degradation and since alfalfa is a good source of protein we can conclude that the diet was being metabolized. Based on the results from this study we conclude that alfalfa crumble diets did not result in elevation of the metabolic stress markers examined. Further studies have been performed to evaluate the immune response of molting hens fed an alfalfa diet.

CHAPTER IV

IMMUNE AND STRESS PROTEIN RESPONSE IN MOLTING LAYING HENS CHALLENGED WITH *SALMONELLA* ENTERITIDIS AND FED ALFALFA CRUMBLE DIETS

Introduction

Molting occurs naturally in the avian species usually at the end of a breeding season. For commercial egg producers this will mean a period of unproductivity because the molt is incomplete in commercial laying hens and they continue to lay eggs at low rates for a prolonged period of time (Berry, 2003). In order to obtain a more uniform molt within the flock commercial egg producers in the US historically have used feed removal to induce a molt. Conventional feed withdrawal programs involves the removal of feed, water (no more than 3 days) or both from the hens and also reducing the photo-period in the houses to 8 h lighted period or less. The removal of feed should last for a period which is long enough to cause the complete involution of the reproductive tract (Berry, 2003).

Induced molting by feed withdrawal increases the susceptibility of hens to *Salmonella* Enteritidis (SE) infection (Holt, 2003; Ricke 2003). *Salmonella* infection in laying hens is a serious food safety concern, since infection of a bird could lead to the production of SE positive eggs after molt (Humphrey et al., 1993). While poultry is not the only source of SE to humans, it is by and large the most common when the source of the infection can be traced, and eggs are the primary source (Patrick et al., 2004). A variety of different physiological mechanisms contribute to the hens' reduced resistance

to pathogens during molting. Among the investigated mechanisms that are known to cause decreased resistance include alterations in the intestinal microflora (Corrier et al., 1997; Holt and Porter, 1992; Savage, 1989), intestinal physiology (Barnes et al., 1979; Corrier et al., 1997; Duke, 1986; Holt, 1993), and host defenses (Ben-Nathan et al., 1981, 1977; Holt, 1992a, 1993). During an induced molt, immunosuppressive effects on T-cell immunity (Holt, 1992b, 1993) and humoral immunity (Ben-Nathan et al., 1981, 1977) have been reported and even with the massive invasion of inflammatory cells to the site of infection, molted hens are still more susceptible to infection. Wolford and Ringer (1962) observed that molting influenced the differential white blood cell counts by causing a reduction in lymphocytes numbers and increasing heterophil numbers in the peripheral blood.

An acute phase response is mediated early during inflammation, and is manifested by an increase in the production of serum proteins from the liver (Godson et al., 1995). These serum proteins, specifically acute phase protein (APP) are released into the bloodstream by a variety of stimuli including inflammation (Thomas and Schrieber, 1985; Fleck, 1989; Jamieson et al., 1992), bacterial infection (Morley and Kushner 1982; Pfeffer and Rogers, 1989) and endotoxin exposure (Takahashi et al., 1995). Holt and Gast (2002) observed that birds that were experimentally infected with *S*E and were molted or not molted, exhibited substantially higher APP levels than non-infected birds. They concluded that serum APP; specifically α_1 acid glyco-protein (AGP) levels can be effective and rapid indicators of infection and can be useful in tracking the infection status of a flock.

Animal welfare concerns of reduced welfare of hens that are force molted by feed deprivation has led to investigations into alternative methods of inducing a molt. Different methods have been examined for their potential to induce a molt instead of using feed deprivation and the methods usually incorporate some form of nutritional imbalance to induce molts (Bell, 2003; Park et al., 2004). Low energy high fiber diets such as wheat middlings, cotton seed meal and jojoba meal have been shown to successfully induce molts (Biggs et al., 2004; Davis et al., 2002; Arnouts et al., 1993) and limit *S*E colonization (Seo et al., 2001). Our previous research has evaluated the potential of alfalfa to serve as the primary dietary component for molt induction and retention of egg production during the second egg laying cycle (Donalson et al., 2005; Landers et al., 2005a,b; Woodward et al., 2005). When used to induce a molt in laying hens, alfalfa limited *S*E colonization and infection during the molt (Woodward et al., 2005; McReynolds et al., 2005; 2006). Due to the concerns of immunosupression during an induced molt, the present study was undertaken to assess the capability of alfalfa to reduce immunosupression during an induced molt. The specific objectives were to evaluate the following immune responses to SE infection; serum antibody, intestinal mucosal antibody, bile antibody, changes in the H: L ratios and also to evaluate the levels of AGP concentration in the serum of SE infected hens on an alfalfa crumble diet fed during an induced molt.

Materials and Methods

Experimental Design

Experiments 1 and 2 were conducted using 250 Single Comb White Leghorn hens (SCWL) over 50 weeks old, which were obtained from a local commercial laying flock. Twelve hens were used in each of 6 treatments in trial 1 and 10 hens were used in each of 6 treatments in trial 2. The hens were placed in wire layer cages and were given free access to water and un-medicated corn-soybean meal based mashed layer ration that met National the Research Council recommendations for nutrients (1994). The alfalfa crumble diet was considered to be high in crude fiber (24. 1%), with moderate crude protein (17. 5%) and low in metabolizable energy (1200 kcal/kg; NRC, 1994). Feed and fecal samples (1 g) were collected and examined for salmonellae. Samples were cultured in tetrathioninate broth and on brilliant green agar[1] (BGA) plates as was previously described by Andrews et al. (1995). All the hens and feed used in both trials tested negative for *Salmonella.*

The hens were allowed to acclimatize in the cages for a period of two weeks after which 12 (10 in the case of trial 2) hens were randomly assigned to 6 treatment groups designated as followed; (1) feed withdrawal SE+ (FW+), (2) full fed SE + (FF+), (3) 100% alfalfa crumble SE + (ALC+), (4) feed withdrawal SE - (FW-), (5) full fed SE -, and (6) 100% alfalfa crumble SE - (ALC-). The molt procedure used to induce the molt was a modification by Holt (1993) which was previously described by Brake et al. (1982). Treatment diets were applied to each treatment group on d 1 of the molt, at the

same time feed was removed from the feed withdrawal hens. Treatment diets were administered for 12 days, the period of time the FW hens were deprived of feed. Hens in all the treatment groups were given water *ad libitum*. The SE positive hens were all placed in the same room, while the SE negative hens were placed in a separate room. Foot baths were placed at the doors of all the rooms in the facility and non-infected hens were cared for before the infected hens each day. The hens in all the treatment groups were exposed to 8 h light: 16 h dark photo-period one week before changing the diets and removing feed from the FW hens. This light schedule continued for a 12 day period after which the experiment was terminated.

Bacterial Strain

For this experiment a primary poultry isolate of *S.* Enteritidis (phage type 13A) from the National Veterinary Services Laboratory, Ames, Iowa, selected for resistance to novobiocin and nalidixic acid (NO and NA) in the USDA-ARS facility, College Station, TX, was used. The media used to culture the resistant isolate contained 25 µg of NO and 20 µg of NA per ml. The culture was prepared from an overnight culture previously transferred three times in trypticase soy broth. The challenge inoculum was prepared by serially diluting the culture in sterile phosphate buffered saline (PBS) to a concentration of approximately 10^6 cfu per ml. The cfu of the challenge inoculum was confirmed by plating on BGA plates. On d 4 of the molt, all the hens in groups 1, 2 and 3 were challenged by crop gavage with 1ml of inocula containing approximately 10^6 cfu of NA and NO resistant SE. The challenge dosage was slighter higher than the $5.6x10^4$ cfu dosages reported by Holt (1993) to be the mean

infectious dosage for *S*E in molted hens. Groups, 4, 5 and 6 were not challenged with SE.

Blood Collection

Twenty-one gauge, 1.5 inch needles were used to collect approximately 8 ml blood from the jugular vein in the neck of 12 birds which were randomly selected on at the beginning of the study to obtain the baseline data. Eight hens were bled in a similar manner from each treatment group on days 2, 5, 9 and 12 of the molt. Seven ml was placed in 10 ml non-heparinized blood collection tubes and the remaining 1 ml was placed in EDTA tubes to be used for smearing microscope slides to count for heterophil to lymphocyte ratio. The plasma was separated by centrifugation at 2500 rpm X 15 min, the clear supernatant plasma from each sample were collected and placed in plastic vials and were stored at -20° C until chemical analysis could be conducted. One drop of blood was placed on microscope slides to be counted for heterophil to lymphocyte ratio (H: L). The serum was placed into aliquots of 200 µl and stored at -20° C until it was used for ELISA assays. Serum samples were diluted in sterile PBS to 1: 320.

Intestinal Sample Collection and Preparation

Following euthanasia, samples were collected from the ileum and the ceca. The samples were immersed in ice cold Hank's Balanced Salt Solution (HBSS) containing 500 IU/ml of penicillin and 500 µL of streptomycin (Zigterman, et al., 1993). The gut contents were removed by gently flushing each tissue section with HBSS. Each tissue sample was cut into pieces weighing 0.3 g and subsequently cut in three smaller pieces and finally washed with HBSS containing 500 IU/ml penicillin and 500 µl streptomycin.

The tissue samples were suspended in 6 ml of RPMI 1640, supplemented with 100 IU/Ml of gentamicin and 40 mM HEPES buffer. The suspensions were subsequently centrifuged for 5 mins (300 x g). Samples were incubated at 41° C, 5% CO_2, and 95% air in 12-well culture plates for 16 h. After incubation, the samples were placed in 200 µl aliquots and stored at -20° C until they were used for ELISA assays to measure antigen specific IgA. The intestinal media samples were diluted in PBS (pH 7.4) 1: 2.

Bile Sampling

During necropsy, approximately 1 ml of bile was aspirated from the gall bladder of each hen using a 22 inch gauge X 0.5 inch needle with a 2 ml syringe. The samples were placed into micro centrifuge tubes and spun at 15,000 rpm for 30 min at 4°C to separate the larger proteins. The samples were stored at -20°C until needed and at that time samples (100 µl) were diluted to 1: 250 in PBS (pH 7.4) for ELISA.

Indirect ELISA

An indirect ELISA was performed on the serum, intestinal and bile samples that were obtained from the birds. Lipopolysaccharide (LPS) at a concentration of 50 µg: 10 mg was used to coat the 96-well plates with 190 µl of the solution pipetted in each well on the plates. The plates were subsequently allowed to incubate overnight and the following day they were rinsed four times with PBS Tween (pH 7.4, 0.5% Tween 20). The samples from the serum, intestinal media and bile were added (190 µl of each) to the wells using the dilutions mentioned previously. After incubation and rinsing, horse radish peroxidase-conjugated goat anti-chicken IgG (for the serum samples) and IgA (for the bile and intestinal samples) were added to each well. The plates were again incubated

for 1h and rinsed before 150 µl of 1-step™ Turbo TMB was added to each well. After 20 mins, the reaction was stopped by adding 50 µL of 1 M sulfuric acid. The plates were read for absorbance by a multi-well plate reader (Spectra Max, Microplate Spectrophotometer, Sunnyvale, CA) at a wavelength of 450 nm.

AGP Assay

Serum levels of AGP were evaluated using a commercially available radial immunodiffusion tray (Carditech Services Inc. Louisville, KY). The agarose trays were impregnated with anti-sera specific for chicken AGP. Each tray had ten wells cut into the agarose for sample application. Each kit contained two standard samples; a low of 250 µg/ml and a high of 1000 µg/ml of AGP. Of these standards, 5 µl were placed into individual wells on the tray as the results would later be used to provide data for generating a standard curve. The other 8 wells were filled with 5 µl of the test samples, placed in a moisture chamber, and incubated overnight at 25° C. The interaction of the migrating AGP from the standards and the serum samples through the agar with the anti-AGP that was present in the matrix was indicated by the formation of a ring. The concentrations of AGP in the samples and standards were directly correlated with the diameter of the rings, assuming the higher the concentration of AGP in the sample, the larger the diameter of the ring that would be formed. The diameters of the two standards' concentrations values were measured using a measuring gauge provided in the kit. The values were plotted on semi-logarithmic paper provided in the kits with the concentration of AGP on the horizontal axis and the diameter on the vertical axis. A line

was drawn between the two points and served as the reference curve for the concentrations of the remaining samples on the tray.

Statistical Analysis

Intestinal and bile IgA data were determined by analysis of variance using the general linear models procedures. Significant differences ($P \leq 0.05$) were separated using Duncan's multiple range tests and statistical analysis software. The H: L, APP and serum antibody data were summarized in each treatment and means of each parameter over the 12 d period were analyzed using a repeated measures design. Proc GLM (SAS version 8.3, SAS Institute Inc., USA, 2001) was used with treatment, time, and treatment by time interaction and individual hens nested within treatment as the factors. Chicken nested within treatment was the error term used to test for treatment effects. When significant ($P \leq 0.05$) treatment by time interactions was found, means were compared using Least Significant Difference.

Results and Discussion

Heterophil: Lymphocyte (H: L) Ratio

Significant ($P < 0.001$) treatment by time effect was observed in H: L ratios in both trials 1 and 2 (Figure 4.1). In trial 1 all the treatment groups exhibited similar ratios up to d 2 of the molt ($P \geq 0.05$). In trial 2, the ALC hens exhibited an increase in H: L ratio on d 2 after which an increase in the H: L ratios was observed for both FW groups, this increase was observed in both trials and the response was not observed in the ALC hens or the FF hens. On d 4 SE was administered to groups 1, 2, 3, the day after an increase in the H: L ratio was observed in the FW- and FW+ hens (0.32

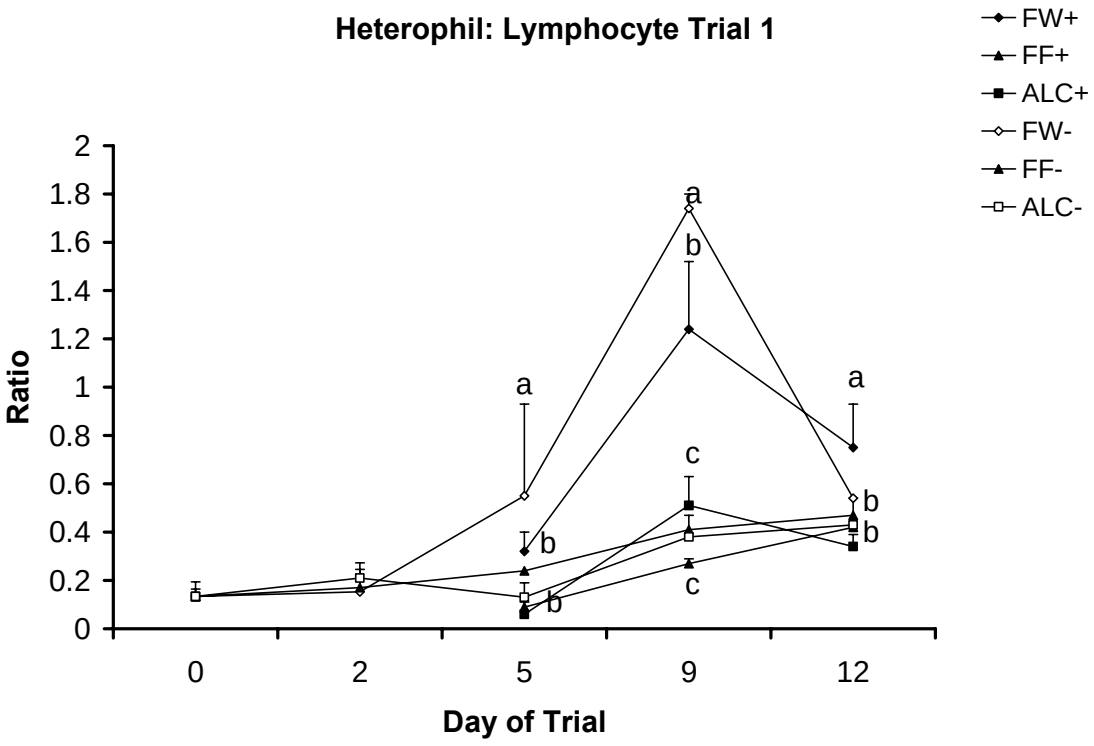


Figure 4.1: The effects of alfalfa diets on heterophil: lymphocyte (H: L) ratios in *Salmonella* Enteritidis (SE) challenged laying hens during molt in trial 1 and trial 2. FW+= Feed withdrawal SE positive hens, FF+= Full fed SE positive hens, ALC+= Alfalfa crumble diet SE positive hens. FW-= Feed withdrawal SE negative hens, FF-= Full fed SE negative hens, ALC- =Alfalfa crumble diet SE negative hens. Treatments with the same letter are not significantly different at $p \leq 0.05$.

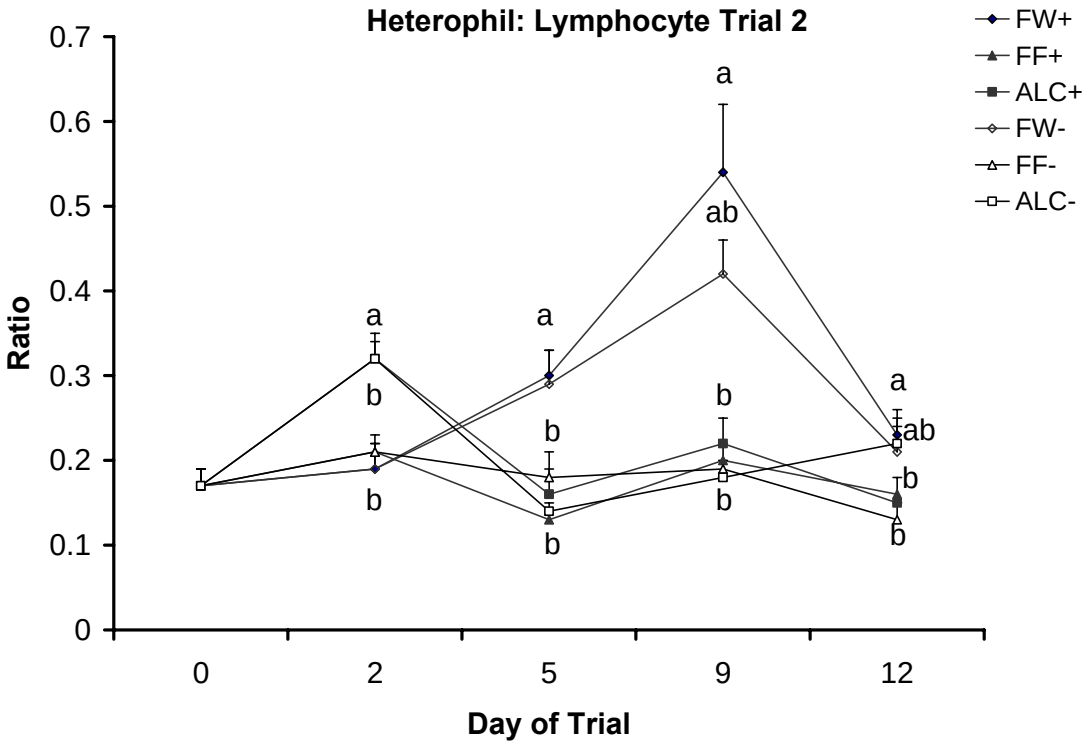


Figure 4.1: Continued

and 0.55 for trial 1 and 0.30 and 0.29 for trial 2 respectively), the increase observed on d 5 was significantly higher than the ratios for the other groups ($P \leq 0.05$). The ALC hens exhibited a ratio of 0.06 for the ALC+ hens and 0.13 for the ALC- hens in trial 1 and 0.16 for the ALC+ hens and 0.14 for the ALC- hens in trial 2, these results were not significantly different ($P \geq 0.05$) from the FF+ hens (0.09 and 0.13) and the FF- hens (0.24 and 0.17) in trials 1 and 2. On d 9 a peak in the H: L ratio was observed in both trials 1 and 2 for both FW groups. The H: L ratio for the FW+ hens in the trials were 1.24 in trial 1 and 0.54 in trial 2, while the FW- hens exhibited ratios of 1.74 in trial 1 and 0.42 in trial 2. These ratios were significantly different from the other treatment These ratios were significantly different from the other treatment groups ($P \leq 0.05$). Again the amount of heterophil that both ALC groups produced was not statistically different ($P \geq 0.05$) from the FF groups in both trials. On d 12, a dramatic reduction in the H: L ratios were observed in the FW hens in both trials, however, the ratios were still significantly higher ($P \leq 0.05$) than the other treatment groups in trial 1 but not significantly different from the ALC- group in trial 2. It has been reported that increases in the H: L ratio in hens is an indicator of chronic stress based on increases in corticosterone levels (Gross and Siegel, 1983; Gross, 1989; Maxwell, 1993; Maxwell et al., 1992b; Siegel, 1995) and Lane (1987) suggested that changes in the levels of the avian heterophil could be used as the window to the state of birds' health. The findings in the current study were consistent with reports by other researchers (Davis et al. (2000) who reported increased H: L ratios and increased corticosterone levels when hens experienced restricted food access. Kogut et al. (1999) also observed a significant rise in

the number of circulating heterophils within two days of feed withdrawal which remained throughout the feed withdrawal period. They also reported that the highest rise in heterophil occurred during the first week of the molt with a dramatic reduction by the end of the molt. The results from these trials are also consistent with studies reported by Alodan and Mashally (1999) who observed significantly higher circulating heterophil on d 5 of a molt, when they compared FW hens with non-molted control hens. They concluded from their results that the FW hens were under more stress than the control hens. In the majority of the avian species, lymphocytes are the most abundant leukocytes found in circulation (Maxwell and Robertson, 1998). In poultry, approximately 59% of the total white blood cells are lymphocytes and approximately 27% are heterophil. Brake et al. (1981) reported that recrudescence and lymphocytic repopulation usually accompanies reproductive quiescence in molting hens and Alodan and Mashaly (1999) found that peripheral lymphocytes decreased and lymphocyte populations changed during the fasting period of a molt. Jones (1989) found that fasted and frustrated Brown Leghorn pullets exhibited increased H: L ratios. Contrary to what was observed in the current study, Maxwell and others (1990) investigated broilers that were subjected to prolonged feed restriction, they found that the H: L ratios of these birds were not significantly altered when compared to birds that were fed *ad libitum,* they concluded that birds became habituated to the effects of prolonged feed restriction. During the early phase of feed restriction, the avian heterophil responds to a hormonal surge of corticosterone (Maxwell et al., 1991, 1992a, b; Savory et al., 1992; Hocking et al., 1993, 1994, 1996), however, this increase is not physiologically sustained; therefore the

heterophils are no longer recruited to the site of infection and resulting in a reduction of the ratio.

Serum Antibody Production

Significant treatment by day effect was observed for serum IgG levels in both trial 1 ($P < 0.03$) and trial 2 ($P < 0.002$). The results (Figure 4.2) revealed that on the day after the SE was administered to the hens (d 5), the FW+ hens produced less ($P \leq 0.05$) IgY than the all the other treatment groups, this was observed in both trial 1 and 2. The antibody absorbance levels for the FW+ hens was approximately 0.69, while all the other groups exhibited antibody absorbance levels of 0.81 or greater. There were no significant differences ($P \geq 0.05$) between the ALC+ hens and the FF+ hens. In trial 1 on d 9 a reduction in antibody absorbance levels was observed in all the treatment groups. In trial 2 the FW+ hens they produced significantly less ($P \leq 0.05$) IgY (0.65) on d 9 than the ALC+ hens (0.79) and the FF+ (0.75). No significant differences ($P \geq 0.05$) were observed between the ALC+ hens and the other treatment groups on d 9 in trial 2. In both trials 1 and 2 on d 12, an increase was observed in amount of IgY produced by the FW+ and ALC+ hens. The FW+ hens exhibited significantly higher ($P \leq 0.05$) antibody absorbance levels in trial 1than all the other treatments with absorbance levels of 0.91 while the FF+, and ALC+, had absorbance levels of 0.64 and 0.80 respectively, these groups were not significantly different ($P \geq 0.05$), no significant differences were observed among treatment groups on d 12 in trial 2.

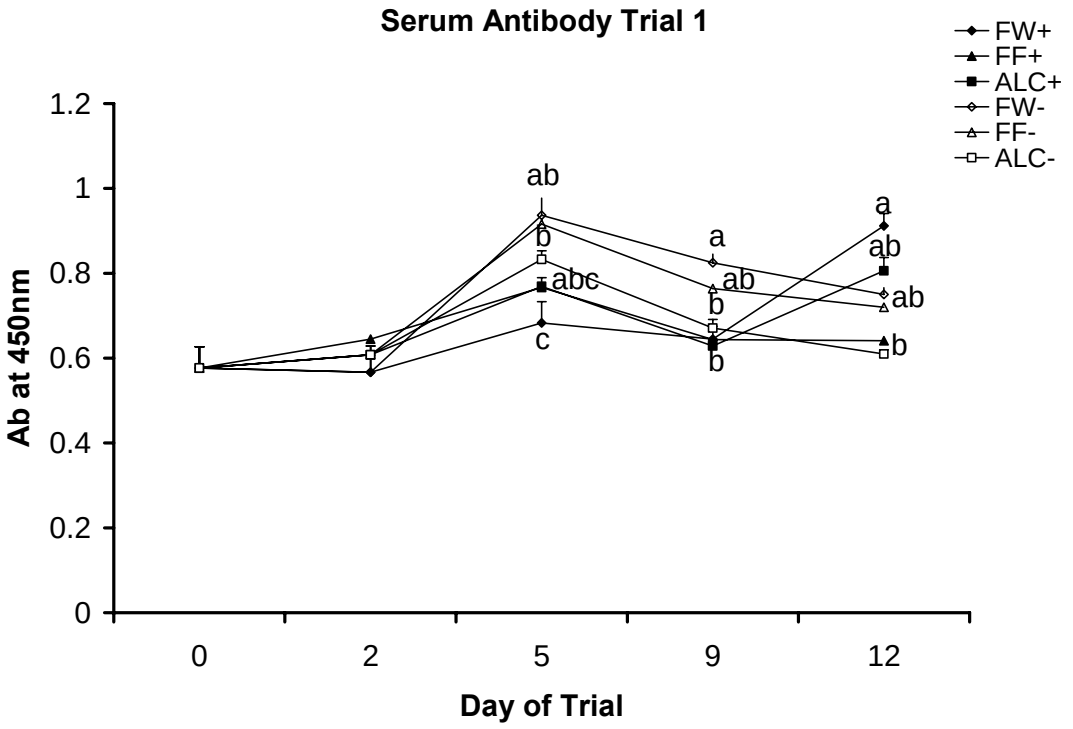


Figure 4.2: The effects of alfalfa diets on serum antibody production in *Salmonella* Enteritidis (SE) challenged laying hens during molt in trial 1 and trial 2.
FW+= Feed withdrawal SE positive hens, FF+= Full fed SE positive hens, ALC+= Alfalfa crumble diet SE positive hens. FW-= Feed withdrawal SE negative hens, FF-= Full fed SE negative hens, ALC- =Alfalfa crumble diet SE negative hens. Treatments with the same letter are not significantly different at $p \leq 0.05$.

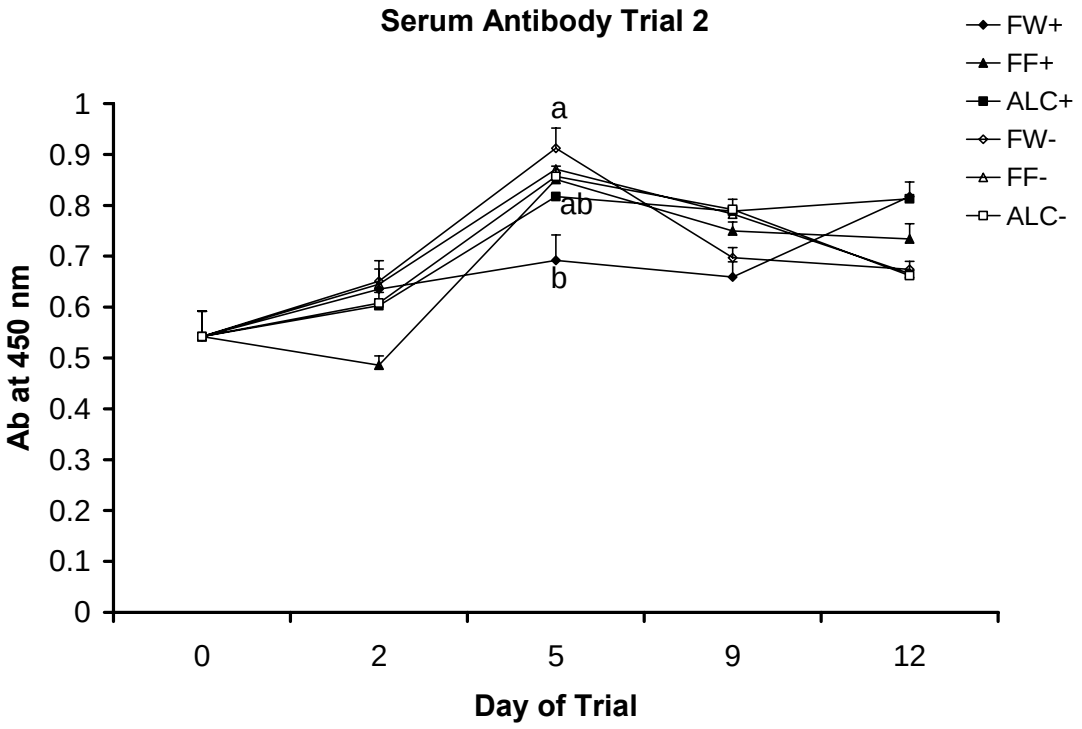


Figure 4.2: Continued

Holt (1992a) observed significant reduction in antibody response to *Brucella abortus* in molting hens on d 5 but no other effects were observed. They concluded that molting exerted minimal effects on the humoral system. Ben-Nathan et al. (1980) previously reported that birds challenged with sheep red blood cell or *Staphylococcus aureus* and deprived of food for 48 h significantly reduced antibody titers when compared to challenge fed birds. In this study the FW+ hens exhibited reduced levels of circulating IgY early in the molt but later increased towards the end of the 12 day fast, this was not observed in the FF+ or the ALC+ hens indicating that feed deprivation did have a negative effect on humoral response early in the fast. Holt (1992a) observed a reduced number of B-cell observed in birds on a fasting induced molt while Desmidt et al. (1998) stated that humoral immunity contributed to the elimination of SE from the gut of poultry.

Intestinal and Bile Antibody Response

The FW+ hens exhibited significantly higher levels ($P \leq 0.05$) of ileum IgA antibody absorbance levels than the other treatment groups in trial 1 but no significant differences ($P \geq 0.05$) were observed between the ALC+ hens and the FF+ hens. In trial 2 no significant difference ($P \geq 0.05$) in the ileum IgA antibody absorbance levels among the FW treatment groups and the ALC treatment groups (Table 4.1). The FW+ and FW- hens yielded IgA absorbance levels of 1.43 and 1.35 respectively while the ALC+ and ALC- hens exhibited IgA absorbance levels of 1.29 and 1.09 respectively. There were no significant differences ($P \geq 0.05$) between the two FF groups and the ALC- group was not significantly different ($P \geq 0.05$) from the FF- group. However, the cecal IgA

production for the FW+ group was significantly higher ($P \leq 0.05$) IgA absorbance levels than the other treatment groups in trial 1 while in trial 2 the FW+ hens yielded significantly higher levels of IgA in the ceca than the FF hens and the ALC hens. Measurements of the bile antibody absorbance levels production in trial 1 indicated that there were no differences among the treatment groups. In trial 2 all SE + groups had higher IgA production with absorbance levels of 1.50 (FW+ hens), 1.51 (FF+ hens), and 1.51 (ALC+ hens). This was significantly different ($P \leq 0.05$) from the FW- and FF- hens (1.42 each), but not ($P \geq 0.05$) from the ALC- hens (1.49). It was reported by Holt (1992a) that induced molting had significant effects on circulating T lymphocytes in hens early during a fast, this in turn could have a significant impact on any immune response that requires T cell subpopulations. Nencione et al. (1983) and Lillehoj (1991) stated that intestinal intraepithelial lymphocytes can potentially protect against intestinal pathogens. Brito et al. (1993) also observed significant increases in antibody titer in the bile, intestinal mucosa and the serum of young chicks that were infected with *S.* Typhimurium. Our bile antibody production results were also consistent with results observed by Seo et al. (2002) who observed optical density values from SE positive bile that were significantly different ($P \leq 0.05$) from those of negative samples. Zigterman et al. (1993) also observed no difference in the cystic bile of chicken infected with *Eimera tenella*. They concluded that this could have been the result of relatively low concentrations of specific IgA in the presence of high concentrations of non- specific IgA. Mockett and Rose (1986) also were unable to show significant responses in bile due to high background. The increased levels of IgA observed in the ALC- hens could

Table 4.1: The effects of alfalfa diet on intestinal and bile IgA secretion in *Salmonella* Enteritidis (SE) challenged laying hens during molt in trials 1 and 2

Treatment[1]	Ileum	Ceca	Bile
		Trial 1	
FW+	0.19±0.07[a]	0.10±0.03[a]	0.24 ±0.06
FF+	0.11±0.03[c]	0.06±0.01[b]	0.17 ±0.03
ALC+	0.12±0.05[cb]	0.06±0.01[b]	0.15 ±0.02
FW-	0.18±0.06[a]	0.06±0.01[b]	0.15 ±0.05
FF-	0.12±0.04[c]	0.05±0.01[b]	0.16 ±0.03
ALC-	0.14±0.02[b]	0.06±0.01[b]	0.19 ±0.02
		Trial 2	
FW+	1.43±0.18[a]	0.89±0.18[a]	1.50±0.03[a]
FF+	0.52±0.14[c]	0.37±0.10[bc]	1.51±0.003[a]
ALC+	1.29±0.26[a]	0.50±0.08[bc]	1.51±0.02[a]
FW-	1.35±0.19[a]	0.62±0.05[ba]	1.42±0.01[bc]
FF-	0.72±0.09[bc]	0.25±0.06[c]	1.42±0.04[c]
ALC-	1.09±0.20[ba]	0.26±0.02[c]	1.49±0.01[ab]

[a-c] Means within columns with no common superscript differ significantly ($p<0.05$). Means within columns represents antibody titers in different segments of the intestine of hens in the treatments groups.

[1]FW+= Feed withdrawal SE positive hens, FF+= Full fed SE positive hens, ALC+= Alfalfa crumble diet SE positive hens. FW-= Feed withdrawal SE negative hens, FF-= Full fed SE negative hens, ALC- =Alfalfa crumble diet SE negative hens.

be the due to the presence of saponins in the alfalfa. Saponins are steroids glycosides that possess anti-inflammatory and antioxidant properties (Rao and Gurfinkel, 2000).

AGP Response

Significant ($P < 0.006$) treatment by day effect was observed in the study (Figure 4.3). In trial 1 on d 2 we observed no significant difference ($P \geq 0.05$) 2 between the ALC treatment and the other treatment groups, however, there were significant differences ($P \leq 0.05$) between the FW hens and the FF hens. On d 5 of the molt the FW hens and the ALC hens produced similar ($P \geq 0.05$) quantities of AGP and were significantly higher ($P \leq 0.05$) than the FF hens. On d 9 in trial 1 all treatments produced significantly different ($P \leq 0.05$) amounts of AGP with the FW hens producing more than the other two groups and the ALC hens producing more than the FF hens. On d 12 of the molt in trial 1 the FW hens produced significantly more ($P \leq 0.05$) AGP than the ALC and the FF hens which were not significantly different ($P \geq 0.05$). In trial 2 along with the infected groups, non-infected groups were included. There were no significant differences ($P \geq 0.05$) among the treatment groups on d 2 of the molt. On d 5 in trial 2 we observed no significant differences ($P \geq 0.05$) between the molted infected and the FW+ hens. The FF+ and FF- hens were not significantly ($P \geq 0.05$) different from each other, or significantly different ($P \geq 0.05$) from the two alfalfa groups. The non-molted groups were however significantly different ($P \leq 0.05$) from the FW+ and the FW- hens. The ALC+ and the ALC- hens were not significantly different from the FF+ hens but they were different from the FW- hens.

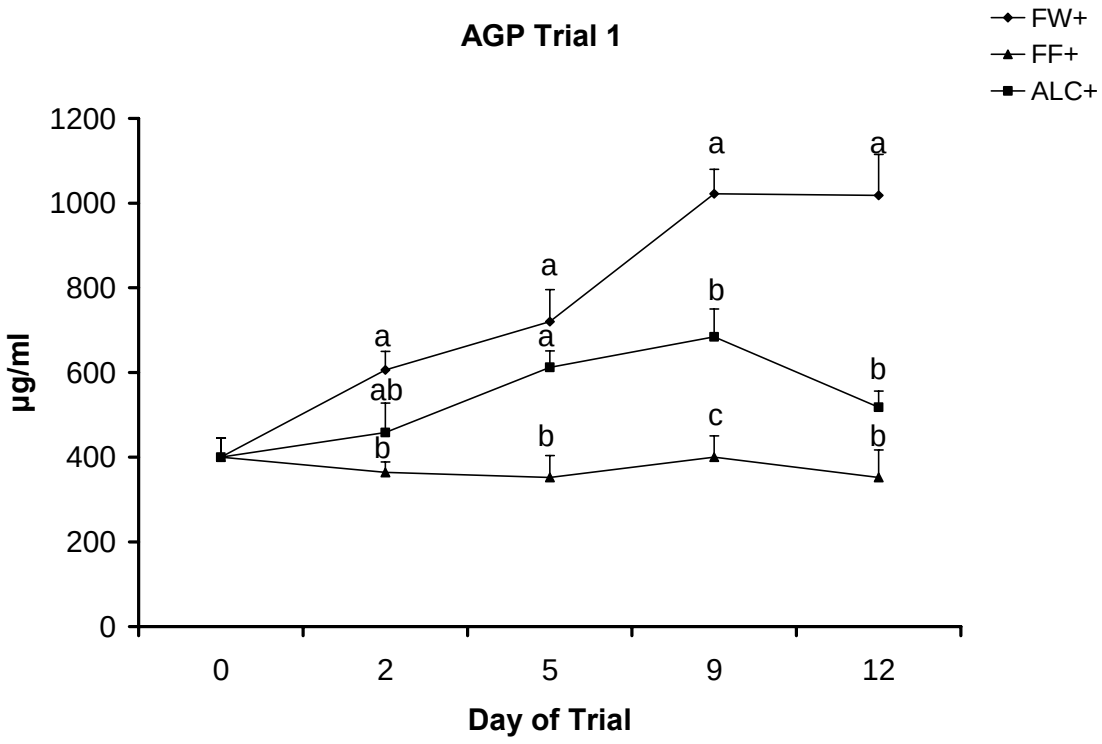


Figure 4.3: Serum α_1-acid glycoprotein (AGP) levels during an induced molt of *Salmonella* Enteritidis (SE) challenged laying hens fed an alfalfa diet in trial 1 and trial 2.

FW+= Feed withdrawal SE positive hens, FF+= Full fed SE positive hens, ALC+= Alfalfa crumble diet SE positive hens. FW-= Feed withdrawal SE negative hens, FF-= Full fed SE negative hens, ALC- =Alfalfa crumble diet SE negative hens. Treatments with the same letter are not significantly different at $p \leq 0.05$.

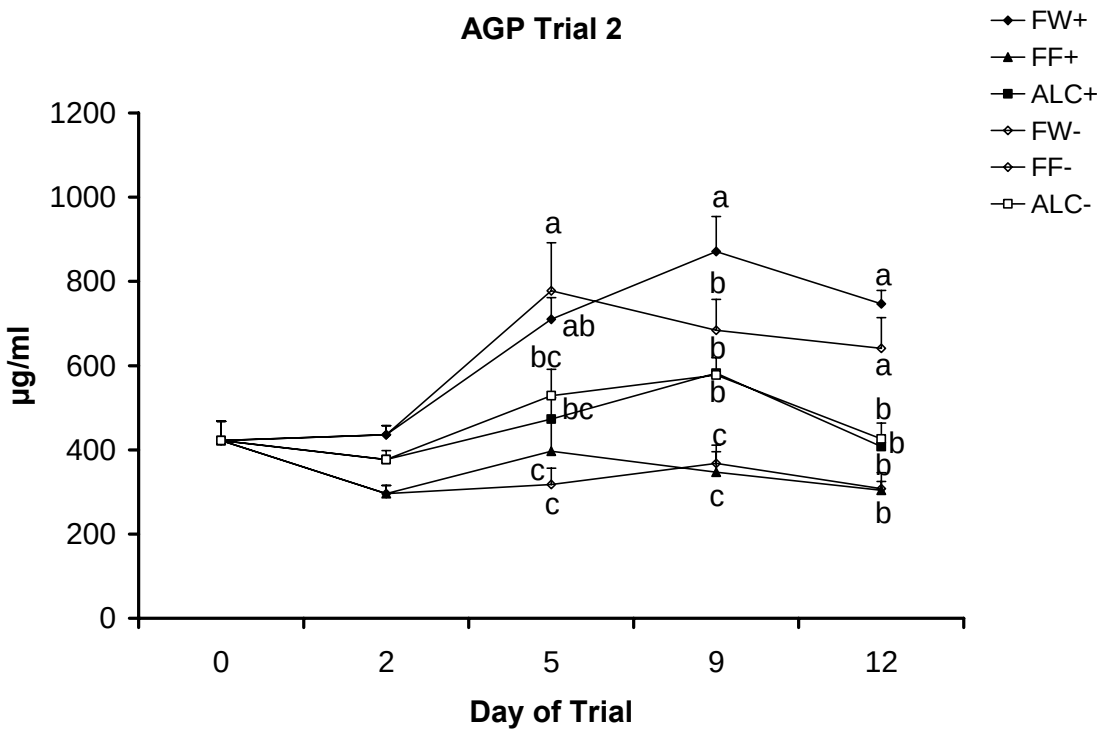


Figure 4.3: Continued

Although Holt and Gast (2002) observed no significant differences between molted infected and non-molted infected hens in two of four trials, they did observe numerical differences with the molted infected hens producing higher concentrations of AGP than the non-molted infected hens they also observed no significant differences among the treatments on d 1 and 3 post challenge but saw an elevation in the AGP serum levels of the molted infected hens on d 8 and d 10 post challenge. These results were similar to what we observed on d 9 of the molt when the FW+ hens had significantly higher titers of AGP than the other treatments. Similarly on d 12 of the molt both the FW+ hens and the FW- hen had significantly higher AGP levels than the other treatment groups, while the ALC hens did not differ from the FF hens.

In conclusion the infectious state of laying hens during an induced molt is a factor in their immune response, generally, the immune response of the SE- hens during this trial were typically lower than the SE+ hens. Our findings suggest that based immunological indicators such as H: L ratio, an alfalfa crumble diet can limit the increase in heterophilia during the molt that is occurs in hens molted by feed deprivation, this was true whether or not hens were infected with SE. Increased heterophilia can cause reduced function of the heterophil (Kogut et al., 1999) resulting in immuno-suppression and reduced resistance to infections such as salmonellosis which can be transmitted to humans via poultry meat and eggs. The reduced serum antibody response that was observed in the FW hens at the beginning of the molt was not observed when hens were fed alfalfa crumble during an induced molt however, it appears that the infectious state of the hens played a role since the SE- hens did not display reduced

antibody levels. Likewise the ALC hens did not exhibit the increased antibody titers in the ceca that were observed in the FW hens. In the current study we observed that alfalfa molt diets reduced the level of AGP in the serum to levels similar to non-molted full fed hens. This would indicate that the induction of a molt using alfalfa molt diets can reduce inflammation from an SE outbreak furthermore even in the absence of an infectious agent such as SE the alfalfa diet effectively reduced the levels of AGP in the serum during an induced molt. This corresponds with our previous work that alfalfa based diets effectively induce a molt with post-molt performance comparable to that of feed deprived hens and limit SE infection (Woodward et al., 2005; McReynolds et al., 2005, 2006). However, it is pertinent to note that other factors such as endotoxin exposure and bacterial infection can cause a raise in the levels of serum AGP (Thomas and Schreiber, 1985; Pfeffer and Rogers, 1989; Takahashi et al., 1995). The use of an alfalfa diet during an induced molt could have a significant impact on food safety by reducing immuno-suppression and alleviating animal welfare concerns of reduced resistance to disease due to starvation.

CHAPTER V

BEHAVIOR OF LAYING HENS ON ALFALFA CRUMBLE MOLT DIETS

Introduction

Commercial laying hens, like most species of wild birds, experience naturally occurring molts (Swanson and Bell, 1975). In the avian species molting usually involves the periodic shedding and replacement of feathers, but is usually incomplete in commercial laying hens and they continue to lay eggs at low rates for a prolonged period of time (Swanson and Bell, 1975). An incomplete molt means a period of un-profitability due to a reduction of egg production and the end of the useful life of a flock (Berry, 2003). One of the largest incurred expenses in the commercial layer industry is that of replacement pullets (Bell, 2003). Commercial egg farmers typically extend the productive laying life of their flock from less than 80 wk to 110 wk or even 140 wk through the use of induced molting (Bell, 2003). After the induced molt, egg production, and egg quality are improved significantly compared to the pre-molt period (eg. Webster, 2003; Bell, 2003; Swanson and Bell, 1975). Molting may be induced by feed withdrawal for up to 10 days (e.g. Christmas et al., 1985), and (or) water withdrawal for two days (North and Bell, 1990), along with a reduction of day-length (e.g. Hembree et al., 1980). Feed and water withdrawal is controversial and has been outlawed in Europe but feed withdrawal has been used for molt induction in the U.S. (Appleby et al., 2004. Park et al., 2004). Feed withdrawal is also problematic because it has been shown in experimental models to increase *Salmonella* Enteritidis colonization and infectivity in the gastrointestinal tract of laying hens (Holt, 2003; Ricke, 2003).

The practice of feed withdrawal has been characterized as a stressful (Beuving and Vonder, 1978) causing a general deterioration of the well-being of animals and usually involves a cascade of physiological adaptive responses (Thaxton and Puvadolpirod, 2000). Duncan and Wood-Gush, (1971) stated that feed deprivation was frustrating and negatively affected the welfare of the bird due to the initial period of fasting to bring about the cessation of lay. Webster (2000) postulated that behavior might be a direct indicator of the well being of hens undergoing feed withdrawal, however, studies that quantified aggressive behavior during induced molts have been contradictory. Aggrey et al. (1990) and Haskell et al. (2000) reported increased aggression in feed deprived hens while Hembree et al. (1980) did not observe this increased aggression. Webster (1995) found no significant differences in aggression between caged hens on a 4 day fast and hens that were not deprived of feed. Webster (2003) stated that although there is no evidence that management programs involving feed withdrawal cause debilitation when properly implemented, there is debate whether or not the hunger involved in feed withdrawal causes suffering.

In light of this, various methods of nutrient restriction that would avoid long term feed withdrawal and the use of dietary additives have been investigated (Berry, 2003, Webster, 2003, Park et al., 2004). Alternative methods for molt induction include feeding low calcium (Breeding et al.,1992) or low sodium diets and high dietary zinc (Berry and Brake, 1985), or high fiber, low energy diets (Donalson et al.,2005; Landers et al., 2005 a, b; Woodward et al., 2005; Biggs et al., 2003, 2004; Seo et al., 2001). Each method is typically employed in combination with a change in the photoperiod, and

usually leads to body weight loss, the cessation of egg production, regression of the reproductive system and induction of a molt (Shippee et al., 1979; Bell, 2003; Park et al., 2004).

Among the high fiber low energy molt diets, alfalfa molt diets have been shown to effectively regress the reproductive system and bring about a rapid return to egg lay at a rate similar to feed withdrawal hens (Donalson et al, 2005; Landers et al, 2005 a, b, Woodward et al., 2005). Alfalfa diets also appear to reduce *S*. Enteritidis colonization in the organs and reduce intestinal shedding of the pathogen during molting when compared to feed withdrawal hens (Woodward et al, 2005, McReynolds et al., 2005, 2006). However, hen behavior during induced molting using alfalfa molt diets has not been investigated. The objective of this study was to evaluate the behavior of hens and compare these behavioral patterns to that of feed withdrawal hens over a 9 day induced molt.

Materials and Methods

Experimental Design

Ninety Single Comb White Leghorn hens approximately 60 weeks old were obtained from a local commercial layer farm. The hens were divided into two groups and placed in floor pens with five broiler birds in each pen, for a period of 2 wks. This was done to train the hens to use the nipple waterers in the battery cages. The hens in each treatment were placed in identical adjoining temperature controlled rooms to prevent the influence of the behavior of one treatment group on another.

The hens were randomly placed on both tiers of two- tier battery cages, with three hens per cage and an allowance of 1100 cm^2 sq ft per bird. Twenty four hens were placed in each room for a two week acclimatization period, during which they were fed a balanced un-medicated corn-soy bean meal based mash layer ration that met the National Research Council requirements for nutrients (NRC, 1994) and water *ad libitum.* The full-fed (FF) group served as the control group and remained on the acclimatization ration. Alfalfa typically contains 17 to 18% crude protein and 24 to 25% crude fiber and has a low metabolizable energy (1200 kcal/kg) when compared to a layer ration with 2,965 kcal/kg (NRC, 1994). Hens in the ALC treatment group were fed alfalfa crumble *ad libitum* for the 9 d trial. The alfalfa crumble was obtained by passing 2 cm long alfalfa pellets through a crumbler that reduced the pellets to approximately 0.5- 0.8 cm. Feed was withdrawn (FW) from the third group for the 9 d duration of the trial. One week prior to the beginning of the trial, the lighting schedule in the three rooms was changed from 16 h light, 8 h dark to 8 h light, 16 h dark. On day 1, only the alfalfa diet was offered to the ALC hens and feed was withdrawn from the FW hens. Eight cages (three hens in each cage) on both the upper and lower tiers in each room were video recorded. Two cameras were mounted approximately 1.2 m away and 30° above the cages in each of the three rooms, with one camera focused on four cages. The video were recorded to a digital multiplexer (Kalatel DVMRe Triplex© eZ Digital Video Multiplexer Recorder, Corvallis, OR). Recordings began at 1200 h each day and ended at 1400 h and two 10 min intervals were analyzed each day from d 0 to d 9 of the trial.

One ten min interval recorded at 1230 h and a second 10 minute observation period started at 1330 h.

Behavior Parameters

The 24 hens were the subjects for the behavior analysis in each treatment. Seven different behaviors were adapted from Webster (2000) to assess the birds' welfare during the trial. Head movement was considered to be the rapid individual head movement of an alert bird. This is suggestive of visual surveillance by a bird of its environment. Feeder activity involved any pecking behavior that was directed towards the feeding trough while the focal bird was close enough to the trough to eat or when the bird had her head in the trough. Preening involved the manipulation of the plumage with the beak. Drinking was considered to be the apparent ingestion of water by pecking from the nipple waterers. Nonnutritive pecking behavior was recorded when hens were pecking at anything other than the feed, which included pecking at the cage floor, sides and their own feet. Walking was defined as the locomotion of the hen involving at least one step. Aggression was recorded as any aggressive pecking behavior directed to another hen either in the same cage or in a neighboring cage. Aggressive behavior was observed as a 0/1 occurrence. The data were in the form of counts at one minute intervals that were then summarized as the daily mean percentage of observations in which the subject was performing a particular behavior.

Statistical Analysis

The average of each activity of the three hens in each cage was summarized and means of each behavior on each of the nine day period were analyzed as cage averages

using a repeated measures design. Proc GLM (SAS version 8.3, SAS Institute Inc., 2001) was used with treatment, time, and treatment by time interaction and individual hens nested within treatment as the factors. When significant ($P \leq 0.05$) treatment by time interactions was found, means were compared using Least Significant Difference.

Results and Discussion

Aggressive Behavior and Egg Production

The study was uneventful and proceeded as planned. There were no mortalities in the any of the three treatments throughout the nine-day molt. Aggressive behavior was not observed in the FF or the ALC hens throughout the 9 d trial. One incidence of aggression between hens in separate cages was observed among the FW hens on d 8 (data not shown). Aggressive behavior was observed by Duncan and Wood-Gush (1971) and Haskell et al. (2000) in frustrated hens. Webster (2000) and Anderson et al. (2004) observed that aggression in food deprived hens declined as the molt progressed. McCowan et al. (2006) observed that hens molted by feed deprivation and hens molted while on a low calorie diet displayed increased aggressiveness during the fast period.

The ALC hens stopped laying on d 6 of the trial the same time as the FW hens (Figure 5.1). The FF hens did not stop laying throughout the trial. This was consistent with Biggs et al. (2004) who observed that hens molted on a wheat middlings diet stopped laying by d 6 of the trial. Previously Seo et al. (2001) observed hens molted with wheat middlings stopped laying on d 7. When Keshavarz and Quimby (2002) induced a molt in laying hens using grape pomace, hens stopped laying by d 4 after the

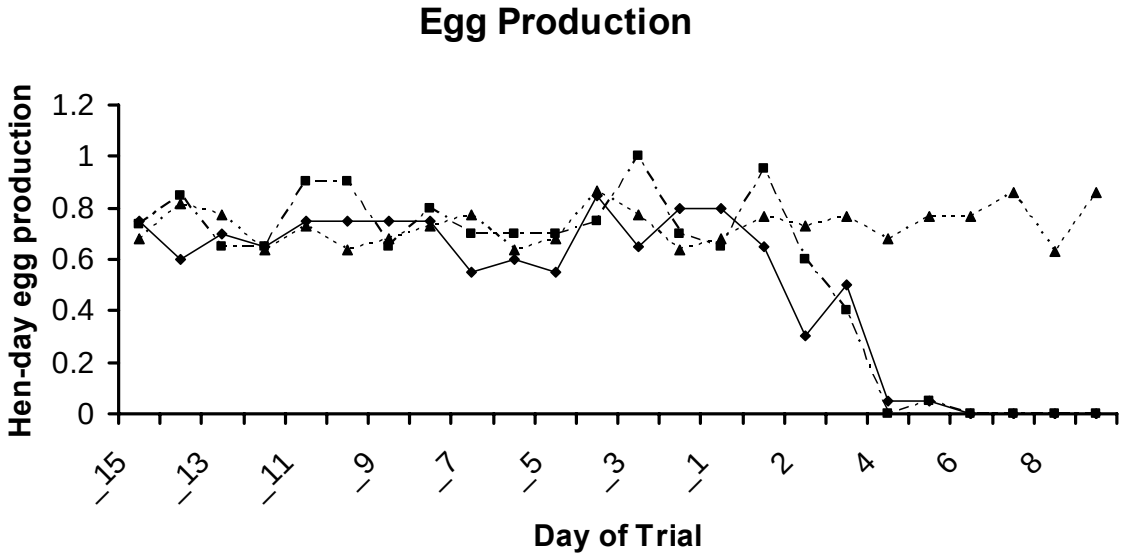


Figure 5.1: Hen day egg production by the three treatments on a daily basis from 2 wk prior molt induction and during the 9 day trial.
The negative numbers represents the pre-trial days.
■= hens fed an alfalfa crumble diet (ALC); ♦ = hens molted by feed withdrawal (FW);▲ = non-molted control hens (FF). N= 24hens per group.

initiation of the molt.

Head Movement

No significant treatment by day effects ($P \geq 0.40$) was observed among the three treatment groups in head movements (Figure 5.2) throughout the 9 d trial. These results suggest that the ALC and FW hens were no less attentive or alert than the FF hens. This was similar to observations by McCowan et al. (2006). However, Webster (2000) observed increased levels of attentiveness among fasted hens, as indicated by increased head movement an indication of their alertness during the molt. Since the hens in the current study were separated based on treatment, the activities of one group would not affect the other. Webster (2000) utilized partitions to separate treatments but they were not isolated from the vocalizations of the control hens.

Walking Activity

Significant treatment by day effects was observed in walking activity ($P \leq 0.001$). On d 1 of the trial the ALC hens spent more time walking (Figure 5.3) than the FW and FF hens ($P \leq 0.03$). On d 2 the FF hens did not differ significantly ($P \geq 0.10$) from the other treatments, however the ALC hens spent more time ($P \leq 0.001$) walking than the FW hens. On d 3 the ALC hens spent more time ($P \leq 0.05$) than the FW and FF hens walking around the cages. On d 4 the ALC hens and the FF hens spent more time ($P \leq 0.03$) than the FW hens walking. The results of d 5 were similar to what was observed on d 3, the FF hens and the FW hens spent less time than the ALC hens walking. While on d 6 walking behavior was similar to d 4, the ALC and FF hens spent more time walking than the FW hens.

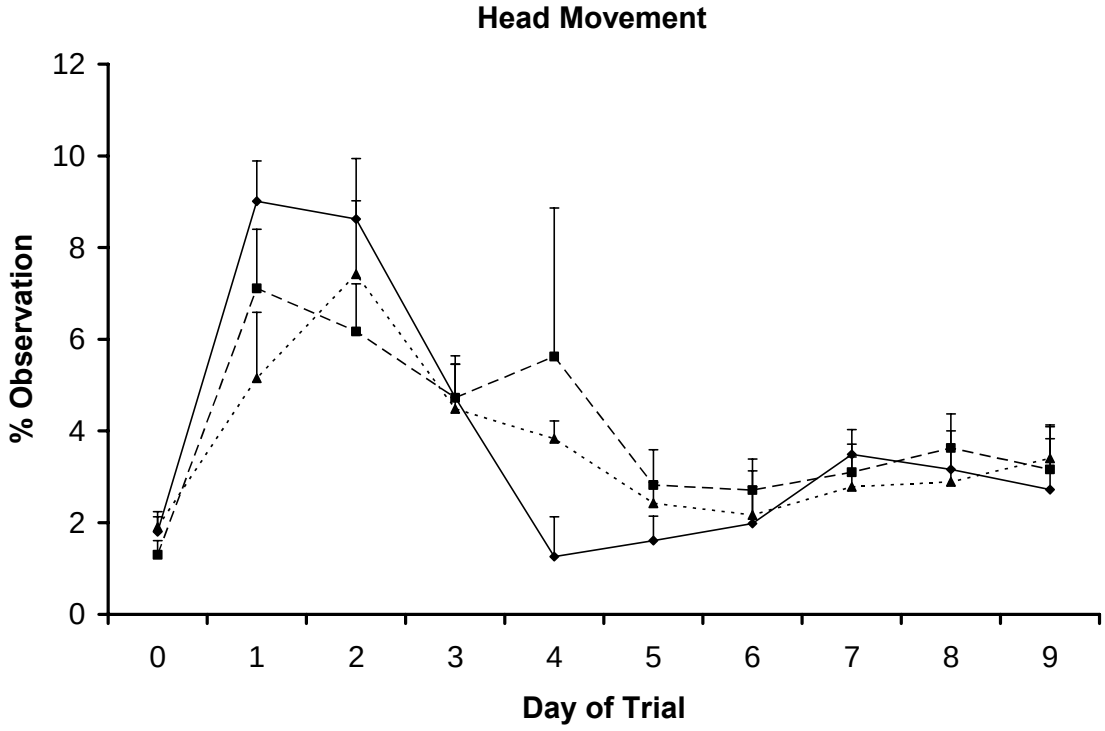


Figure 5.2: Percentage observations of head movement behavior of laying hens during a 9 day induced molt.
■ = hens fed an alfalfa crumble diet (ALC); ♦ = hens molted by feed withdrawal (FW); ▲ = non-molted control hens (FF). N= 24 hens per group. No significant differences were recorded at $p \leq 0.05$.

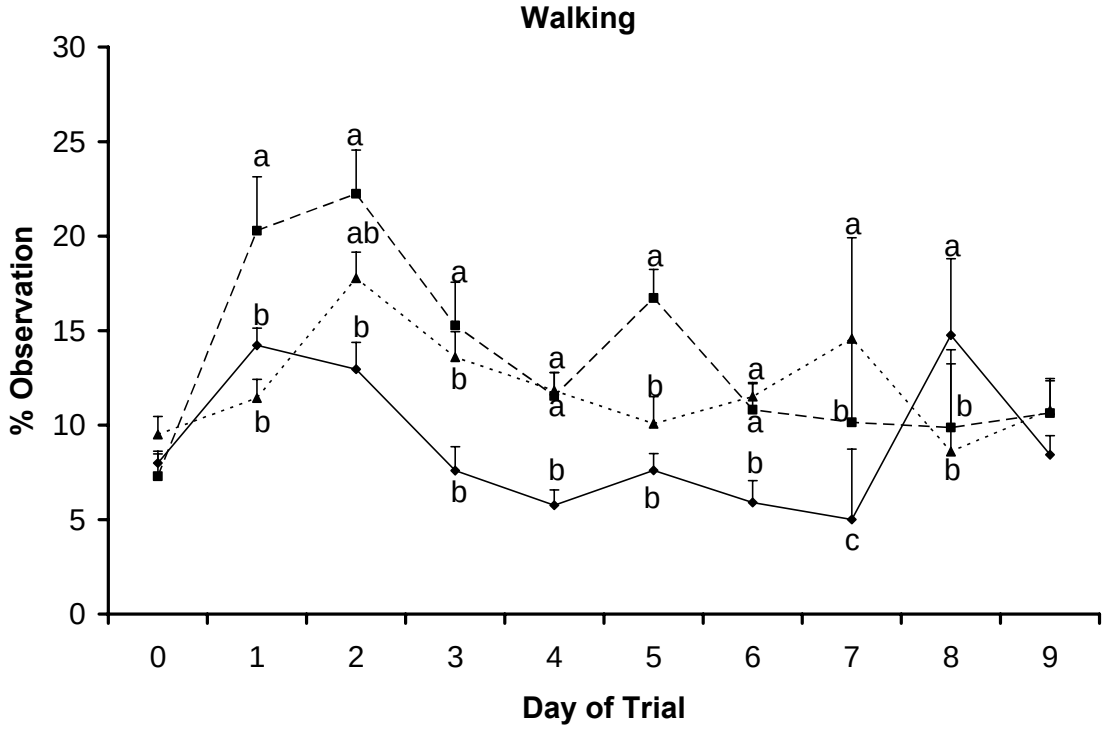


Figure 5.3: Percentage observations of walking behavior of laying hens during a 9 day induced molt.
■ = hens fed an alfalfa crumble diet (ALC); ♦ = hens molted by feed withdrawal (FW); ▲ = non-molted control hens (FF). N= 24 hens per group. Treatments within the same day with different letters are significantly different at $p \leq 0.05$.

On d 7 the FF hens spent more time than the other treatments involved in walking behavior, the ALC hens spent more time than the FW hens, which spent the least amount of time walking. On d 8 the FW hens spent more time walking than the other treatments but there were no differences between the ALC hens and the FF hens. There were no significant differences among any of the treatments on d 9.

Reduced walking activity was observed in the FW hens as the molt period progressed up to d 7 when they exhibited the most walking activity, after this, they returned to a level that was similar to the other treatments. This behavior could be expected because these hens were not being fed and would exist on energy reserves from the liver and adipose tissues (Cherel et al., 1988). The level of reduction observed in the FW was not seen in the ALC hens. During molting most birds, whether in captivity or under natural conditions, decrease all locomotive activities (Murphy, 1996). The significant reductions in locomotor activity that are common during the periodic molt of birds and result in energy savings that can almost compensate for energetic cost due to integumental loss and replacement during a natural molt in captive hens (Beckerton and Middleton, 1983; Lindström et al., 1993).

Preening Activity

Preening activities through the duration of the trial approached significant treatment by day effects ($P \leq 0.06$). The ALC hens exhibited significantly more ($P \leq 0.05$) preening (Figure 5.4) than the FF and FW treatment groups on d 1 of the molt with means of 8.0, 2.4 and 3.5% respectively. There were no significant differences ($P \geq 0.05$) among the three groups in preening on d 2 and d 3 of the trial.

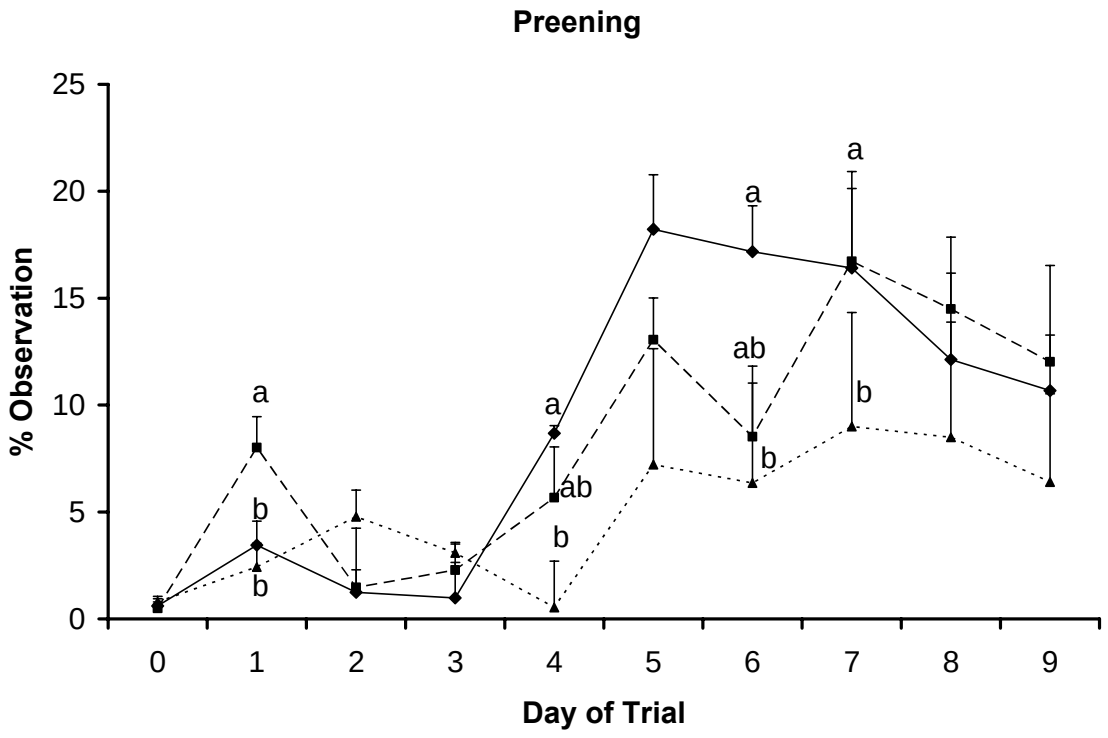


Figure 5.4: Percentage observations of preening behavior of laying hens during a 9 day induced molt.
■= hens fed an alfalfa crumble diet (ALC); ♦= hens molted by feed withdrawal (FW); ▲= non-molted control hens (FF). N= 24 hens per group. Treatments within the same day with different letters are significantly different at $p \leq 0.05$.

On d 4 of the trial the ALC hens did not display significant differences ($P \geq 0.50$) from the other treatment groups however, the FW hens spent significantly more ($P \leq 0.04$) time preening than the FF hens. The differences between the three treatments on d 5 was not significant ($P \geq 0.05$) however, on d 6 the FF hens spent significantly more ($P \leq 0.04$) time preening than the FF hens but the ALC hens were not significantly different ($P \geq 0.05$) from either FW hens or FF hens. On d 7 the FF hens spent less ($P \leq 0.05$) time preening than the other groups but there was no differences between the ALC hens and the FW hens. For the remaining two days of the trial there were no significant differences among any of the treatments.

The FW and ALC hens in the present study were observed spending an increasing amount of time preening up to d 7 of the trial. The FF hens in this trial could also be seen performing preening activity but they spent less time involved in the activity than the FW and ALC hens. The preening behavior of these two groups may be related to loss of feathers during the molt period. Shedding of the feathers was subjectively determined by the first signs of the feathers under the cages on d 8 in the FW treatment and d 9 in the ALC treatment. Preening behavior can be stimulated by integumentary irritations which can be an indication of feather push out (Webster, 2000), as a displacement action in situations of conflict or frustration (Duncan and Wood-Gush, 1972) or as a comfort behavior (Nicol, 1989). This behavior is generally suppressed when hens have less time (Budier, 1996). Webster (2000) observed the FW hens performed more preening behavior than the FF hens on days 8 to 10 but not at other times.

Drinking

Significant treatment by day effects ($P \leq 0.002$) were observed in drinking behavior. The FF hens spent the most time ($P \leq 0.05$) drinking throughout the molt period, followed by the ALC hens and the FW hens (Figure 5.5) during the 9-day trial. The FF hens were drinking significantly more ($P \leq 0.0001$) than birds in other treatments on d 1 while the ALC and FW were not different ($P \geq 0.05$) on this day. Similar results were observed on d 2, 4, 5, 8 and 9. On d 3 and 6 the FW hens spent significantly less ($P \leq 0.05$) time than the other treatments drinking and the ALC hens spent less time ($P \leq 0.05$) than the FF hens drinking behavior on these same days. There were no significant differences ($P \geq 0.05$) between the ALC hens and the other two treatments on d 7 however, the FF hens spent more time drinking ($P \leq 0.05$) than the FW hens.

Drinking behavior of the FW and ALC hens declined from the beginning of the trial to levels that were lower than the FF hens. These reduced levels observed in the FW hens did not return to the levels observed in the FF hens, while the ALC hens exhibited similar amount of drinking as the FF hens on d 7. The reduced drinking behavior observed specifically in the ALC and FW hens could be due to observations made in previous reports that alfalfa fed hens do not eat as much of their diet as the FF hens and no diet was available for the FW hens to consume (Donalson et al., 2005). This is supported by Woodward et al. (2005) who observed that alfalfa fed hens eat significantly less feed than full fed hens. They also reported that full fed hens drank more water than alfalfa fed hens and feed deprived hens. Webster (2000) examined the behavior of hens molted by feed

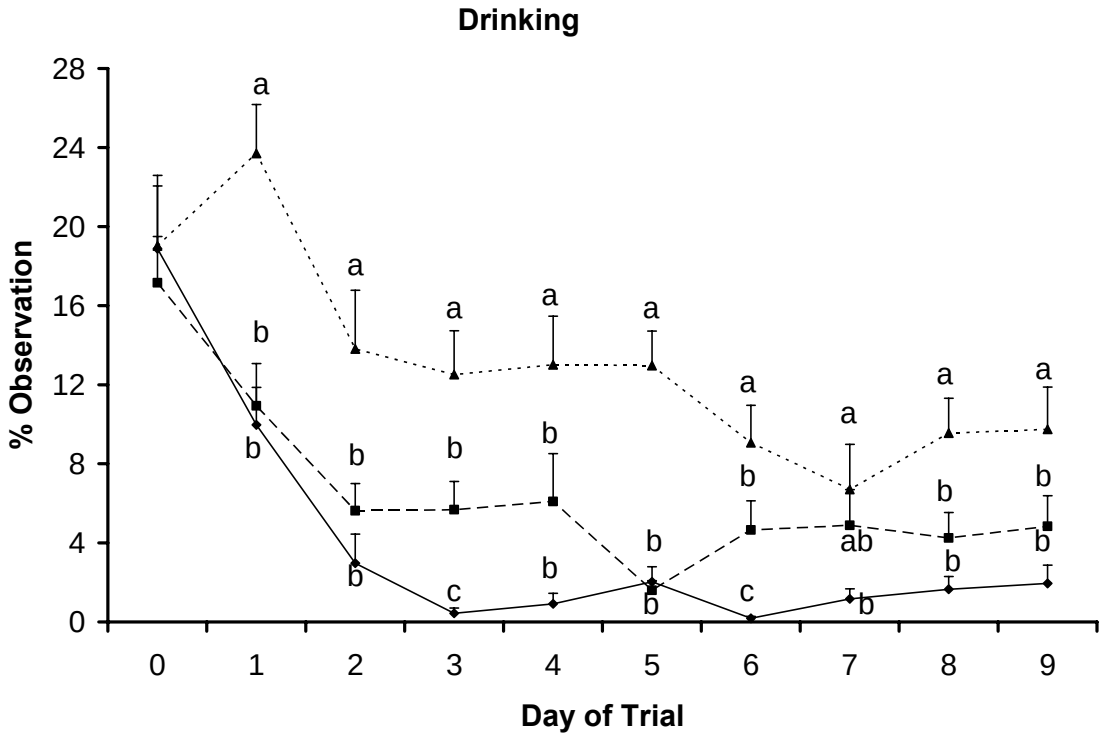


Figure 5.5: Percentage observations of drinking behavior of laying hens during a 9 day induced molt.
■ = hens fed an alfalfa crumble diet (ALC); ♦ = hens molted by feed withdrawal (FW); ▲ = non-molted control hens (FF). N= 24 hens per group. Treatments within the same day with different letters are significantly different at $p \leq 0.05$.

deprivation and observed that the drinking behavior of these hens declined after the first few days of feed withdrawal.

Nonnutritive Pecking

A significant day by treatment effect was observed for nonnutritive pecking. On the first day (Figure 5.6) of the trial (d 1), there was a significant difference ($P \leq 0.001$) between the FW hens and the ALC and FF hens with means of 50.90, 22.10, and 29.80% respectively. There were no differences between the ALC and FF hens on this day. On d 2 and 4, the ALC hens exhibited intermediate behavior with the FW hens spending more time in nonnutritive pecking and the FF hens spent the least amount of time. On d 3 no significant differences ($P \geq 0.10$) were observed between the FW and the ALC hens, but these two treatments spent significantly more ($P \leq 0.0001$) time involved in this activity than the FF hens. The FW hens spent significantly more ($P \leq 0.002$) time on d 4 in nonnutritive pecking than the other groups while the ALC spent significantly more ($P \leq 0.01$) time than the FF hens. A similar pattern was observed d 5 and d 6 with no differences ($P \geq 0.05$) observed between the FW and the ALC hens. On d 7 the FW hens spent significantly more time ($P \leq 0.005$) than the other treatments displaying nonnutritive pecking behavior while the ALC hens spent more time ($P \leq 0.05$) than the FF hens expressing this behavior. On the final two days of the molt there was no difference ($P \geq 0.05$) between the FF (42.45 and 37.79% for d 8 and 9 respectively) and the ALC hens (48.01 and 33.73% for d 8 and 9 respectively), while the FW hens (69.75,

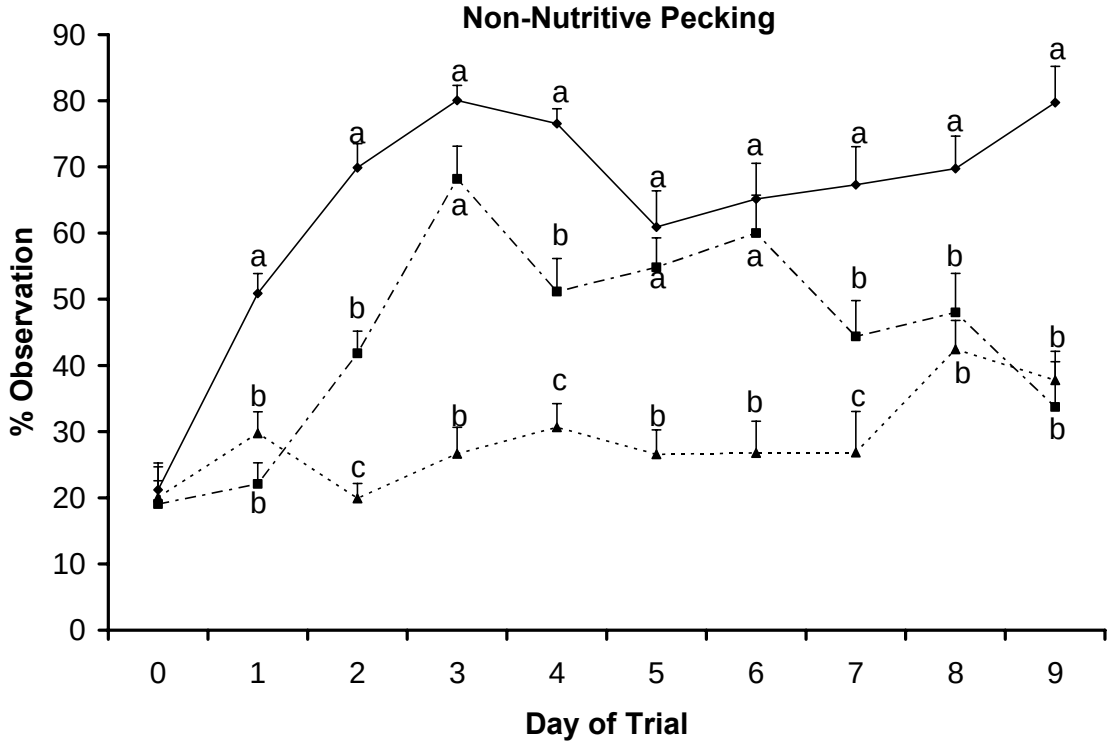


Figure 5.6: Percentage observations of nonnutritive pecking behavior of laying hens during a 9 day induced molt.
■ = hens fed an alfalfa crumble diet (ALC); ♦ = hens molted by feed withdrawal (FW); ▲ = non-molted control hens (FF). N= 24 hens per group. Treatments within the same day with different letters are significantly different at $p \leq 0.05$.

and 79.73% for d 8 and 9 respectively) spent significantly more ($P \leq 0.05$) time in nonnutritive pecking activities.

The increased nonnutritive pecking observed in the ALC hens could have been because of the change of feed since the behavior subsequently declined in these hens after the third day on the diet. The FW hens however, continued increasing levels of nonnutritive pecking behavior up to the end of the 9 d trial. When prevented from performing a specific activity such as eating, hens have a tendency to substitute or redirect one activity with another action (Cooper and Albentosa, 2003). Webster (1995) found an apparent arousal in behavior directed to foraging and feeding and believed that nonnutritive pecking was a typical response of chickens to feed deprivation. Savory and Fisher (1992), and Savory et al. (1992) also observed nonnutritive pecking behavior in egg-type pullets and growing broiler breeders when they were deprived of feed. Savory and Fisher (1992) concluded that nonnutritive pecking was a redirection of a consummatory response from a nutritive stimulus to a nonnutritive stimulus. Recently McCowan et al. (2006) examined the behavior of molting hens on a low-caloric diet and observed that the feed deprived hens exhibited increased levels of cage pecking, the behavior also increased in the non-fast induced treatment from the levels that was observed pre-molt while the non-molted hens did not display increased level of cage pecking. Appleby and Hughes (1991) reported that even in the presence of available food, birds spent most of their day foraging, this could account for the nonnutritive behavior observed in the FF hens.

Feeder Activity

From this study it was not possible to tell if the hens were actually eating the feed or merely pecking at the feeding trough. Because of this a paradox arose where the FW hens were also scored for the amount of time they spent feeding. We interpreted this score not as the amount of time they spent feeding but as the amount of time they went to check the feeding trough. Significant treatment by day effects ($P \leq 0.0002$) were observed for feeder related activity for the 9 day trial, however, on the first day no significant differences ($P \geq 0.50$) were observed in feeder related activities (Figure 5.7) between the FF hens and the ALC hens with means of 27.5 and 31.6% respectively. The FW hens spent 12.5% of their time on d 1 visiting the feeder, which was significantly different ($P \leq 0.01$) from the other treatment groups. On d 2 the three treatment groups exhibited significantly different ($P \leq 0.05$) responses, the FF hens spent significantly more ($P \leq 0.02$) time at the feeder than the other groups and the ALC hens spent significantly more ($P \leq 0.05$) time than the FW hens. On d 3 there were no significant differences between the FF hens and the ALC hens while the FW hens spent less ($P \leq 0.05$) time at the feeder. With the exception of d 5, the FW hens spent less ($P \leq 0.05$) time at the feeder than the other groups for the remainder of the trial. The ALC hens spent significantly less ($P \leq 0.05$) time at the feeder than the FF hens from d 4 to d 8, but by d 9 there were no significant ($P \geq 0.40$) differences between the ALC hens and the FF hens.

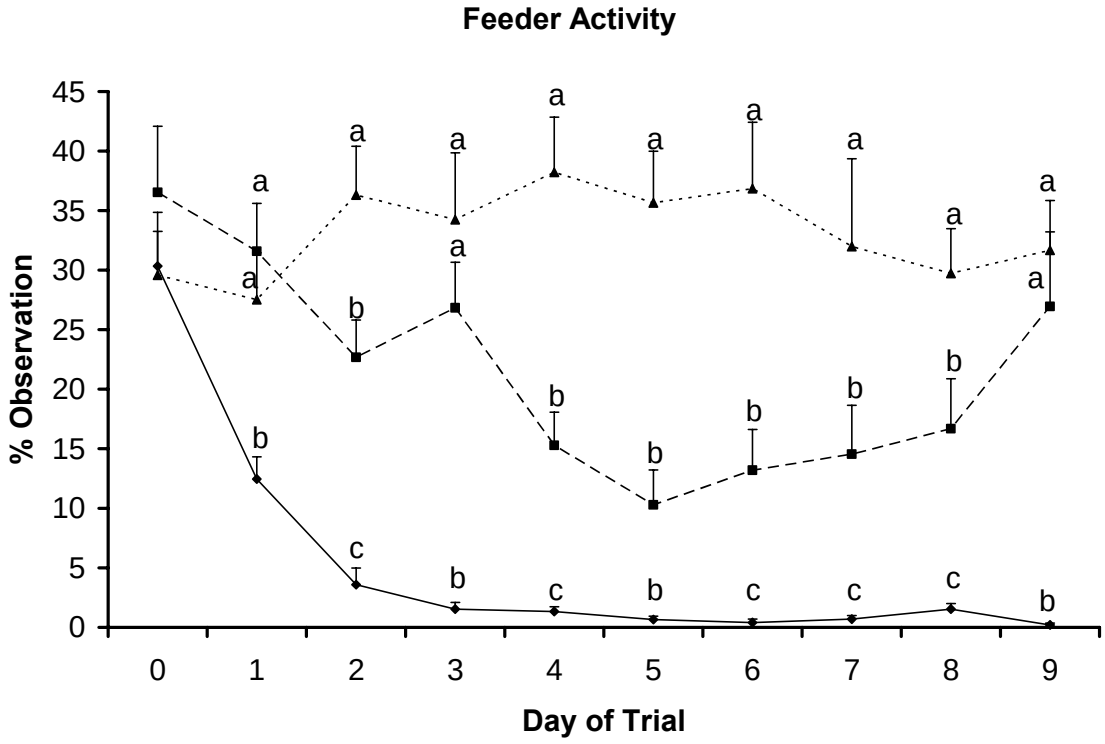


Figure 5.7: Percentage observations of feeder behavior of laying hens during a 9 day induced molt.
■ = hens fed an alfalfa crumble diet (ALC); ♦ = hens molted by feed withdrawal (FW); ▲ = non-molted control hens (FF). N= 24 hens per group. Treatments within the same day with different letters are significantly different at $p \leq 0.05$.

It is apparent that a rapid decline in feeder activity occurs in hens experiencing complete removal of feed. Similar behavior response was observed by Webster (1995, 2000) who reported that hens that were molted by feed withdrawal spent progressively less time visiting the feeder as the molt period progressed. It was also observed in this study that as hens spent less time at the feeder, they spent more time involved in nonnutritive pecking activities; which was consistent with Webster (2000) who observed that a reduction in feeder related activities by FW hens was accompanied by an increase in nonnutritive pecking. Duncan and Wood-Gush (1972) believed that hens needed less than an hour per day to eat sufficient food when a complete ration was provided. It appeared that some adjustment to alfalfa as a feed source occurred as feeder activity initially declined, then increased towards the end of the molt period. However, feeder activity from d 1 and onwards was always greater than FW indicating that the birds were responding to the presence of feed. The reduced feeding behavior in ALC birds compared to FF birds could be the result of reduced intake due to unpalatability of saponins present in alfalfa (Matsushima, 1972; Sen et al., 1998) and/or slow passage rates. This was demonstrated by Sibbald (1979), who reported that alfalfa exhibited the slowest passage rate in chickens requiring more than 24 h to be cleared from the chickens' gastrointestinal tract. He concluded that this gave the birds a feeling of satiety causing them to reduce their intake. When Donalson et al. (2005) evaluated the utilization of different ratios of alfalfa and layer ration for molt induction, they observed that hens fed a diet containing 100% alfalfa meal ate significantly less (82 g/hen) for a 9 d trial when compared to hens fed a diet containing 90% alfalfa plus 10% layer ration

(272.30 g/hen) and hens fed a diet containing 70% alfalfa plus 30% layer ration (409.40 g/hen).

In conclusion, concerns of reduced welfare have been an issue of induced molting by feed deprivation. Behavioral priorities during an induced molt can be an effective way of assessing hens' welfare while they are fasting (Webster, 2000). In this study, the welfare of the hens that were molted using alfalfa was intermediate to the FF hens and the FW hens based on their behavior. The ALC hens did not exhibit displacement behavior, stereotypy or aggression that is indicative of frustration and these hens were given the opportunity to eat even though initially they did not accept the diet. Based on nonnutritive pecking and feeder activity it would appear that once hens adjusted to the alfalfa diet their behavior approximated hens fed a non-molt diet. However, in addition to behavior observations, metabolic and immune indicators need to be monitored to determine whether high fiber diets can decrease physiological stress in hens while they are undergoing a molt and if long-term effects beyond the molt period occur.

CHAPTER VI

LAYING HENS BEHAVIOR TO DIFFERENT ALFALFA-LAYER RATION COMBINATIONS FED DURING MOLTING

Introduction

In the avian species molting usually involves the periodic shedding and replacement of feathers (Lucas and Stettenhein, 1979). Birds undergo a series of molts during their life span. They change at least four different plumages from hatching to their first annual cycle. This includes natal down, juvenile, alternate, and basic plumages (Lucas and Stettenheim, 1972). Molting also involves the regression of the hens' reproductive system resulting in a reproductive quiescence (Berry, 2003). Domestic hens like most species of wild birds experience a naturally occurring molt however, this is usually incomplete and hens continue to lay eggs at low rates for a prolonged period of time (Swanson and Bell, 1975). This would mean a period of un-profitability due to a reduction of egg production and the end of the useful life of a flock (Berry, 2003). The productive laying life of flocks can be extended from less than 80 wk to 110 wk or even 140 wk through the use of induced molting (Bell, 2003). At the end of a laying cycle egg production and quality decline significantly, an induced molt usually improves the hens' performance. After the induced molt, egg production and quality are improved significantly compared to the pre-molt period (Webster, 2003; Swanson and Bell, 1975).

Over the years, a number of different approaches to artificially induce molt in commercial laying hens have been developed. These include feed withdrawal for up to 10 days (Christmas et al., 1985), water withdrawal for two days (North and Bell, 1990)

and photoperiodic reduction (Hembree et al., 1980). The method of feed and water withdrawal is a matter of grave concern to animal advocates and the practice has been outlawed in Europe (Appleby et al., 2004). In light of the animal welfare controversies associated with feed withdrawal molting and the additional problem of increased *Salmonella* Enteritidis infection alternative means to induce a molt that might be considered more humane have been sought in the U.S. (Holt, 2003, Berry, 2003, Ricke, 2003; Park et al., 2004). These alternatives fall in two categories: various methods of nutrient restriction that would avoid long term feed withdrawal and the use of dietary additives (Webster, 2003).

Although egg production parameters have been extensively examined, in many of these alternative approaches, none have examined the behavioral effect of these diets on the hens. Some of these alternative methods have included feeding low calcium (Breeding et al., 1992), low sodium diets and high dietary zinc (Berry and Brake, 1985). Some studies have looked at changes in aggressive behavior during induced molts and the results have been contradictory. Aggrey et al. (1990) found that negative interactions among laying hens increased during feed deprivation in open housing systems but not in battery cages. Hembree et al. (1980) reported that the aggressive behavior of hens in colony cages during feed deprivation did not differ from hens not deprived of feed. Webster (1995) found no significant differences in aggression between caged hens on a 4 day fast and hens that were not deprived of feed while Haskell et al. (2000) found increased aggression in frustrated hens. Alfalfa-layer ration combinations have been shown to be effective for molt induction, reduction of SE infection and retention of

optimal egg production in the second egg laying cycle (Donalson et al, 2005; Landers et al, 2005 a, b, Woodward et al., 2005, McReynolds et al., 2005, 2006). The objective of this trial was to evaluate the behavioral patterns of hens fed different combinations of alfalfa and layer ration and compare these behavioral patterns to that of feed withdrawal hens in a nine day induced molt.

Materials and Methods

Experimental Design

A total of 250 laying hens at approximately 53 weeks old were obtained from a near-by layer unit and used for this trial. Hens were randomly put in five treatment groups and were placed in individual cages (1100 cm^2 sq/bird) in three trial rooms for a two week acclimatization period. During this time they were fed a balanced un-medicated corn-soy bean meal based mash layer ration that met the National Research Council requirements for nutrients (NRC, 1994) and water *ad libitum.* The alfalfa treatment groups were placed in one room, while the FF treatment and the FW treatment were each placed in a separate room. A total of 39 hens were placed in the five treatment groups and 6 hens in each treatment were observed for behavior patterns. The treatments consisted of three alfalfa rations; A90 which consisted of 90% alfalfa meal and 10% layer ration, A80 which consisted of 80% alfalfa meal and 20% layer ration and A70 which consisted of 70% alfalfa meal and 30% layer ration. Alfalfa typicallly contains 17 to 18% crude protein and 24 to 25% crude fiber and has a low metabolizable energy (1200 kcal/kg) when compared to a layer ration with 2,965 kcal/kg (NRC, 1994). Hens in the A90, A80 and A70 treatment groups were fed their respective combination of

alfalfa meal and layer ration *ad libitum* for the 9 d trial. The FF treatment remained on the pre-trial diet and the FW hens were not fed during the 9 d of the trial.

One week before the administration of the diets, the lighting schedule in each of the three rooms was changed from 16h light/8h dark to 8h light/16h dark. Behavior was recorded using a 10 camera Digital Video Recorder Multiplexer system (Kalatel DVMRe Triplex© eZ Digital Video Multiplexer Recorder, Corvallis, OR). Two cameras were mounted approximately 1.2 m away and 30° above the cages of each treatment in each of the three experimental rooms. Two 10 min intervals were analyzed each day from d 0 to d 9 of the trial. Recordings began at 1200 h each day and ended at 1400 h. One ten min interval was taken at 1230 h and second 10 minute observation was taken at 1330 h for detailed analysis.

Behavior Parameters

Eight hens in individual cages were the subjects for the behavior analysis in each treatment. Seven different behaviors were adapted from Webster (2000) to assess the birds' welfare during the trial. Head movement was the rapid individual head movement of an alert bird. This is suggestive of visual surveillance by a bird of its environment. Feeder activity involved any pecking behavior that was directed towards the feeding trough while the focal bird was close enough to the trough to eat or when the bird had her head in the trough. Preening involved the manipulation of the plumage with the beak. Drinking was the apparent ingestion of water by pecking from the nipple waterers. Nonnutritive pecking behavior was recorded when hens were pecking at anything other than the feed, which included pecking at the cage floor, sides and their own feet.

Walking was defined as the locomotion of the hen involving at least one step. Aggression was recorded as any aggressive pecking behavior directed to another hen either in the same cage or in a neighboring cage. Aggressive behavior was observed as a 0/1 occurrence, that is, it either happened or did not happen during the observation time. The data were in the form of counts at one minute intervals that were then summarized as the daily mean percentage of observations in which the subject was performing a particular behavior.

Statistical Analysis

The average of each activity of hens in each treatment was summarized and means of each behavior over the nine day period were analyzed using a repeated measures design. Proc GLM (SAS version 8.3, SAS Institute Inc., Cary, NC, 2001) was used with treatment, time, and treatment by time interaction and individual hens nested within treatment as the factors. Chicken nested within treatment was the error term used to test for treatment effects. When significant ($P \leq 0.05$) treatment by time interactions was found, means were compared using Least Significant Difference.

Results and Discussion

Aggression and Egg Production

Aggressive pecking at neighbors was not significant in this trial. One incidence was observed between cages on d 1 in the A90 and A70 hens and another incidence was observed between cages in the FF hens and the FW hens (data not shown). When Webster (2000) and Anderson et al. (2004) induced a molt by feed withdrawal they observed aggression early during the molt induction. The aggression declined as the

period progressed. This reduction in aggressiveness was not observed by McCowan et al. (2006) when they induced molt by feed deprivation and also by incorporating a low calorie diet. They observed that aggression increased as the period progressed. The low incidence of aggression observed in this trial could result from the hens being housed in individual cages, thereby minimizing the bird to bird interaction usually seen in multi-hen or colony cages.

Egg production data was collected and the A80 hens stopped laying on d 5 of the trial while the FW and A90 hens ceased production on d 6 (Figure 6.1). The A70 hens did not stop egg production up to d 9 of the trial. Donalson et al. (2005) evaluated molt induction and bird performance with different ratios of alfalfa diets, they observed that the A70 hens took an average of 5.75 d to cease egg production while the A90 hens stopped laying 4.92 d and the FW hens stopped 4.42 d (they did not examine A80 hens). The hens in the current study took a longer time to stop laying, however, the trend appear to be the same because the A90 hens stopped laying at the same time or in the case of the A80 hens before the FW hens while the A70 hens did not stop until d 9. This could be due to an incomplete molt because of the higher level of high calorie layer ration that was present in the A70 feed. Donalson also noted that the A70 hens exhibited significantly lower post-molt egg production that was similar to FF hens, while the A90 hens had post-molt egg production similar to the FW hens.

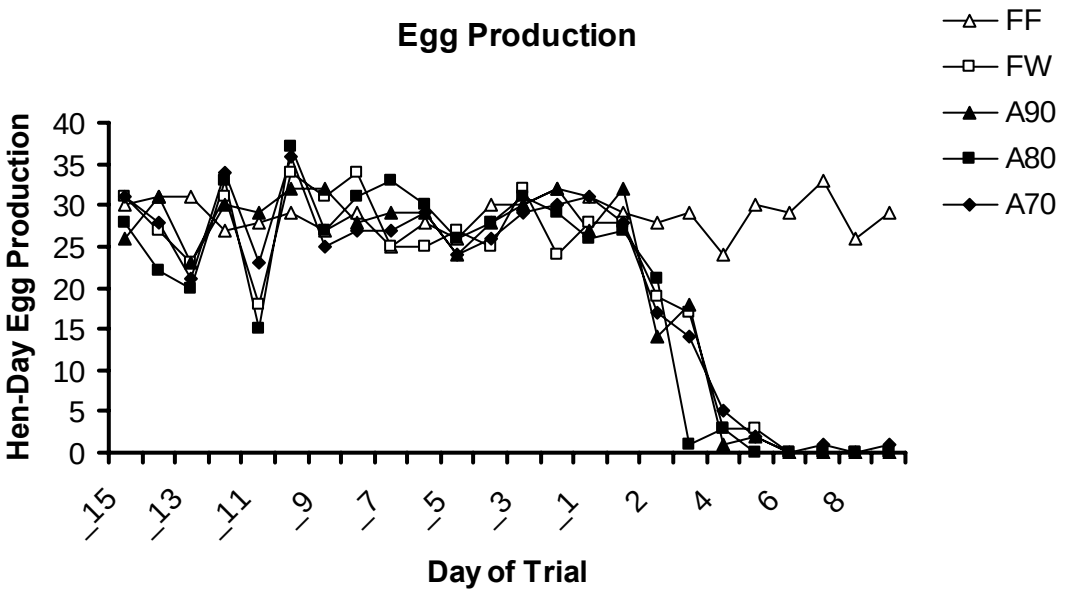


Figure 6.1: Hen day egg production by the five treatments on a daily basis from 2 wk prior molt induction and during the 9 day trial.
The negative numbers represents the pre-trial days.

Walking Activity

No significant ($P > 0.4$) treatment by day effect was observed in walking (Figure 6.2) in this trial, however significant ($P < 0.001$) treatment effects were observed during the 9 d trial. Over this period the FW, A70 and A80 hens spent significantly more time than the than the FF and the A90 hens walking. The FF hens spent more time than the A90 hens walking. In this trial the FW hens (18.64%) had an overall mean that was higher than the FF hens (12.69%) and the A90 hens (5.87%), indicating that they walked more throughout the period than these two groups. The A70 and A80 hens had means of 18.45 and 17.28% respectively which were not significantly different ($P \geq 0.05$) from the FW hens but were significantly different ($P \leq 0.05$) from the A90 and FF hens (data not shown).

The walking behavior observed in this trial was not consistent with what was observed by McCowan et al. (2006) who reported no significant differences among the treatments throughout the molt period. Webster (2000) observed that hens undergoing feed withdrawal spent 40% of their time resting during an induced molt. It would be expected that hens that were feed deprived would reduce their activities in an effort to conserve energy, Murphy (1996) stated that during a molt birds reduce all their locomotive activities.

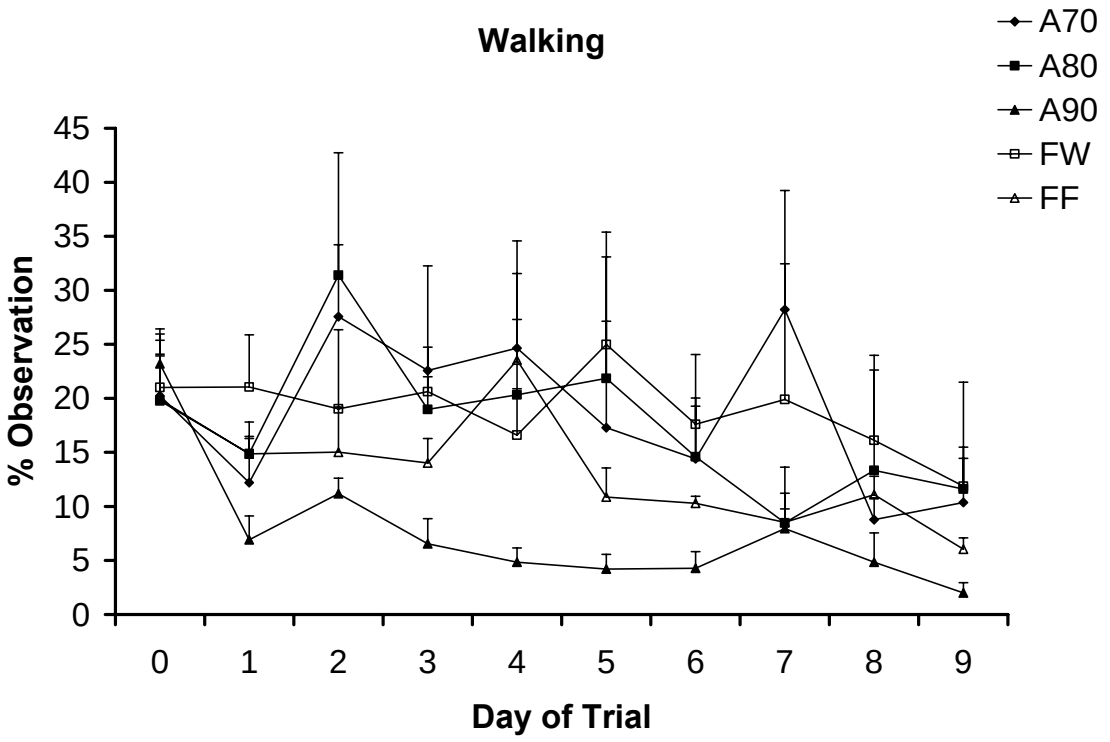


Figure 6.2: Percentage observations of walking behavior of laying hens during a 9 induced day molt.

N= 6 hens per group. No significant differences were recorded at $P \leq 0.05$.

Preening Activity

Preening activity during the 9 d induced molt did not display significant ($P < 0.2$) treatment by day effects. Significant treatment effects were observed on d 5 and 6 (Figure 6.3). On d 5 the A90 hens spent significantly ($P < 0.007$) more time than the FF hens preening, but were not significantly different ($P > 0.05$) from the other treatments. No significant differences were observed among the other treatment groups on d 5. On d 6 the A90 hens displayed significantly more time than the FF and the A70 hens preening while they did not differ from the A90 hens and the A80 and FW hens. On d 6 no differences were observed between the A80, A70, FW and FF hens.

The highest incidence of preening observed during this trial was on d 5, the latter part of the induced molt. For the duration of the trial the A90, A80 and FW hens spent more time preening than the A70 and FF hens, this could have been a result of follicular irritation due to feather push-out because through subjective observations we observed an increase in feathers under the cages of the FW hens on d 8 and under the cages of the A80 and A90 hens on d 9 (data not shown). Shedding was not observed in the A70 hens until d 12 (two days after the treatment diet was removed from the hens). Preening may be performed as a displacement action in situations of conflict or frustration (Duncan and Wood-Gush, 1972). However, Webster (2000) reported that the amount of time that was spent preening followed a quadratic trend that peaked towards the end of a molt induction; he postulated that rather than attributing the increased preening to frustration, it could be attributed to a response to feather push-out.

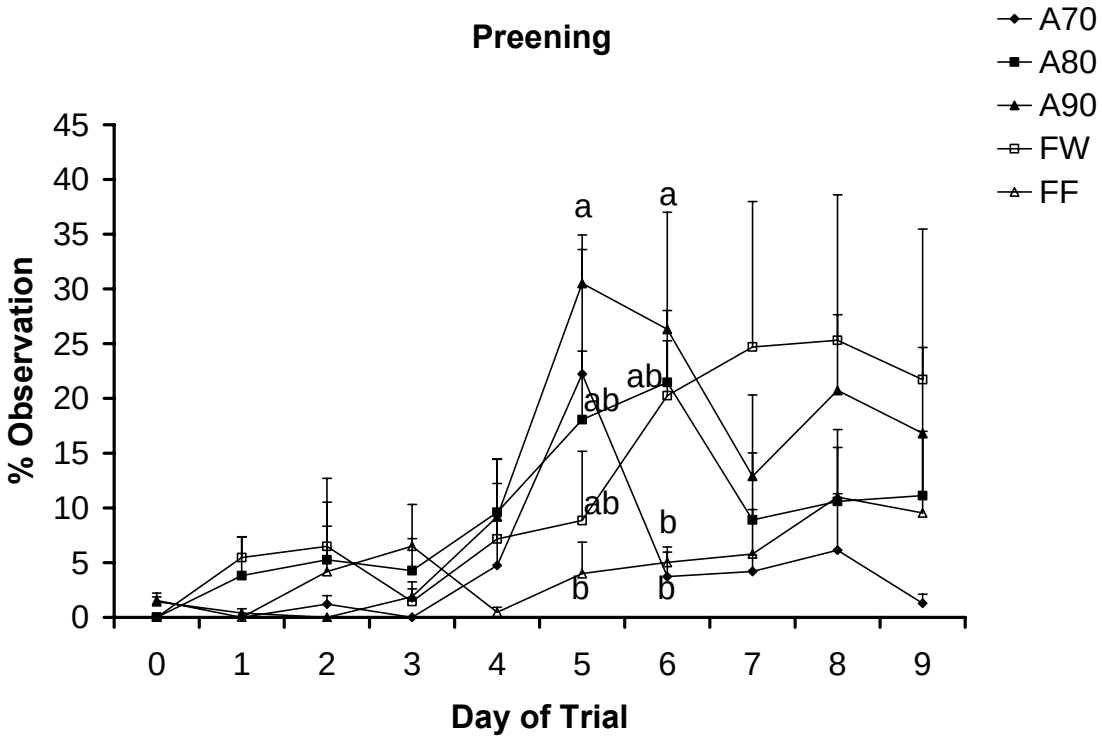


Figure 6.3: Percentage observations of preening behavior of laying hens during a 9 day induced molt.

N= 6 hens per group. Treatments within the same day with different letters are significantly different at $P \leq 0.05$.

Head Movement

Significant treatment by day effects ($P < 0.0003$) was observed in head movement (Figure 6.4) in this trial. On d 1 of the molt the A80 hens spent significantly more ($P \leq 0.01$) time than the other treatments in head movement. There were no significant differences between the A70, FF and FW hens in head movement and the A90 hens spent significantly less ($P \leq 0.002$) time than the other treatments in head movements on d 1. No significant differences ($P > 0.4$)were observed on d 2 among the treatments, however, on d 3, 4 and 9 the A90 hens spent more ($P \leq 0.03$) time performing head movements than the FW hens. The A80, A70, and FF hens did not display significant differences from any other group. No significant differences were observed in head movement among treatments on d 5, 6, 7 and 8.

Most of the head movements observed during the trial were performed during the beginning of the trial. The FW hens spent the least amount of time involved in this behavior for the 9 d period (data not shown). Similar results were observed by Webster (2000) who also noted that the highest frequency of head movement occurred during the first 3 d of feed withdrawal and observed no differences between the FW and non-molted hens during the last days of feed withdrawal. McCowan et al. (2006) evaluated behavior of fast-molted laying hens, low-calorie molted laying hens and non-molted laying hens, they reported no significant differences among treatments in head movements. The alfalfa fed hens were no less attentive during the induced molt because they displayed overall means that were similar to the FF hens.

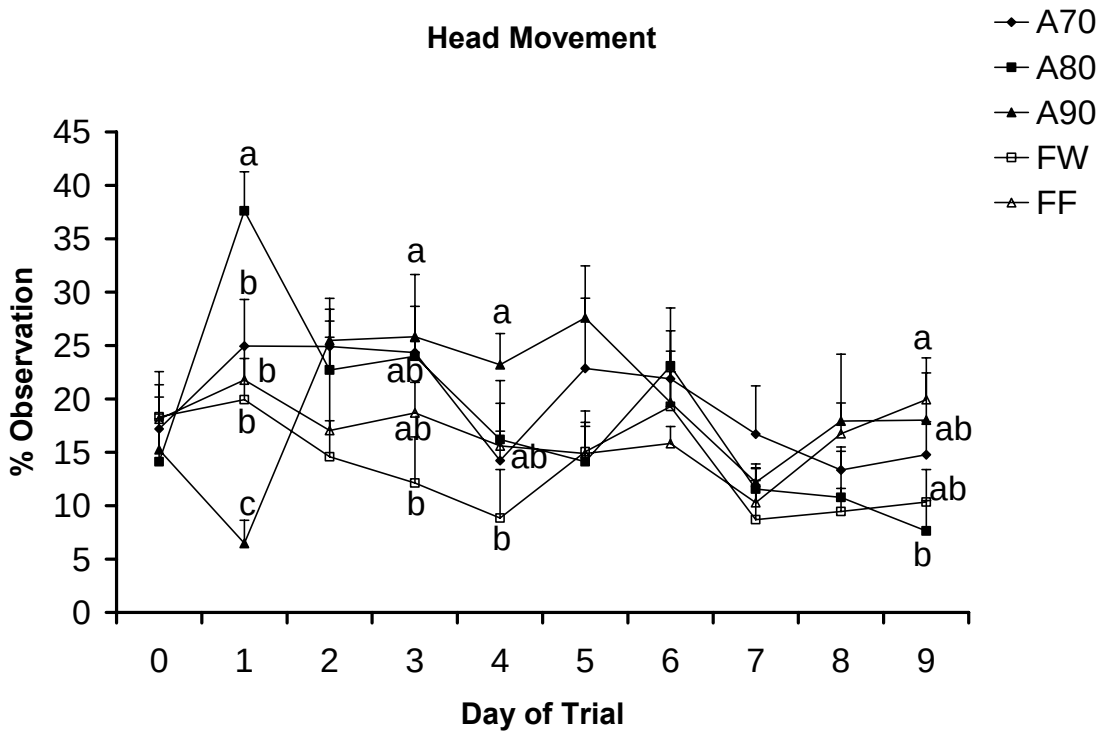


Figure 6.4: Percentage observations of head movement behavior of laying hens during a 9 day induced molt.
N= 6 hens per group. Treatments within the same day with different letters are significantly different at $P \leq 0.05$.

Drinking Activity

Significant ($P < 0.001$) treatment by day effects was observed in drinking activity (Figure 6.5). On d 1 of the trial FF hens spent significantly more ($P \leq 0.006$) time than the A80, A70 and the FW hens drinking. The A90 hens spent significantly more ($P \leq 0.004$) time than the FW hens but did not they differ from the A80, A70, and FF hens. On d 2 the FF hens spent significantly more ($P \leq 0.01$) time than the other groups drinking, there were no significant ($P > 0.05$) difference among the other treatments. No significant differences were observed among the treatments on d 3 and 4, however, on d 5, 6, and 8 the FF hens spent more ($P < 0.0001$) time drinking than the other treatments while there were no differences ($P \leq 0.05$) among the other treatment groups. On d 7 and 9 no differences ($P > 0.05$) were observed among the groups.

The FF hens spent more time than the other groups drinking while the FW hens spent the least amount of time. This behavior is somewhat expected because the FW hens were not eating it would mean they would drink less and since the FF hens which were provided a dry ration, they drank more water. This explanation could be true also for hens on the alfalfa diets because even though we did not evaluate feed intake it appeared that these hens did not eat as much as the FF hens so therefore they would potentially drink less. For the duration of the trial the A80 hens exhibited drinking behavior that was closest to the FF hens. The A90 hens drank less than the A80 hens but they were not significantly different. The A70 hens were observed visiting the waterers the least among the alfalfa fed hens and they were not different from the FW hens.

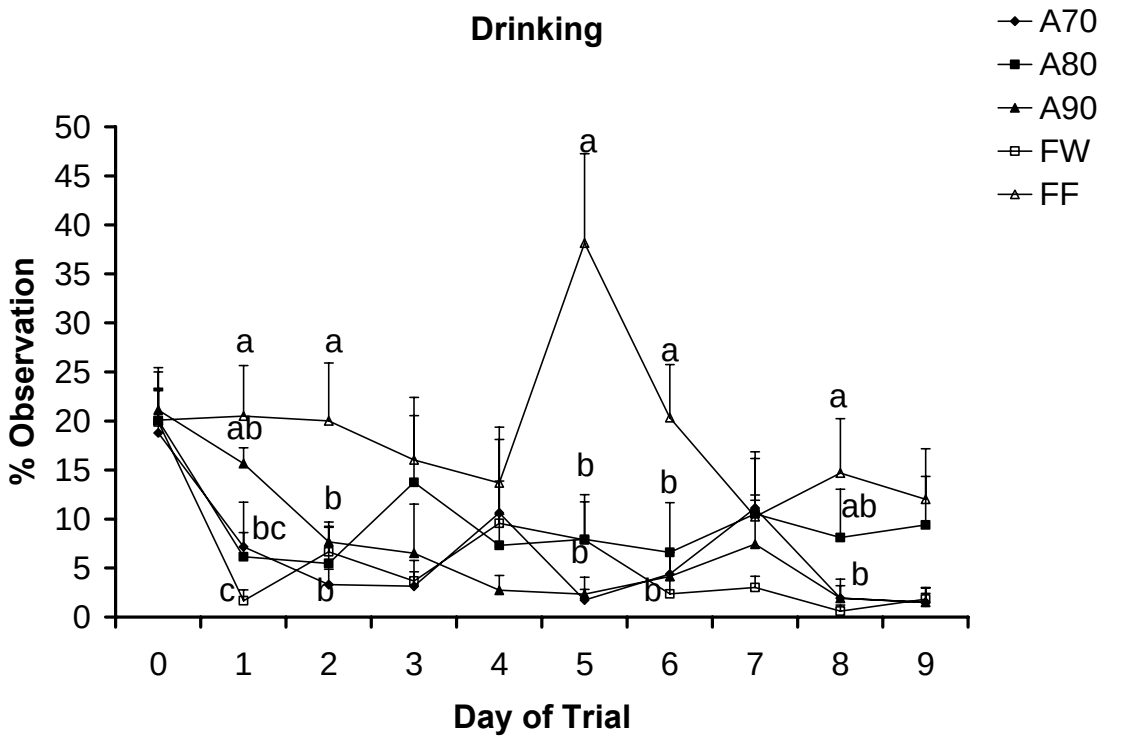


Figure 6.5: Percentage observations of drinking behavior of laying hens during a 9 day induced molt.

N= 6 hens per group. Treatments within the same day with different letters are significantly different at $P \leq 0.05$.

Woodward et al. (2005) evaluated water intake of hens on an alfalfa molt diet and observed that these hens drank 2 fold less water during the molt when compared to the FF hens. The FF hens also drank 2 folds more than the FW hens. Webster (2000) observed that drinking behavior declined in feed deprived hens after the first few days of feed withdrawal.

Nonnutritive Pecking

Significant treatment by day effect did not ($P < 0.10$) occur in nonnutritive pecking behavior (Figure 6.6) however significant ($P < 0.001$) treatment effects were observed in this trial. On d 1 of the trial the FW hens spent significantly more ($P \leq 0.009$) time than the other groups in nonnutritive pecking. On the same day the A70 hens displayed significantly higher ($P \leq 0.05$) levels of nonnutritive pecking than the FF hens but A70 was not higher than the A90 and A80 hens. The A90 and A80 hens did not differ ($P > 0.05$) from the FF hens. On d 2, 3 and 4 the FW hens spent more ($P \leq 0.04$) time performing nonnutritive pecking than the A80 and the FF hens. The A80 and the FF hens were not significantly different ($P > 0.05$) from the A70 and the A90 hens which were not significantly different ($P > 0.05$) from any of the treatments. On d 5 no significant differences ($P > 0.05$) were observed among the groups, however, on d 6 the FW hens spent significantly more ($P \leq 0.03$) time than the FF hens in nonnutritive pecking behavior. The alfalfa fed treatments were not different from any of the other treatments on d 6. On d 7 the A70 hens were not significantly different ($P > 0.05$) from the other treatments, however, the FW hens displayed significantly higher ($P \leq 0.001$)

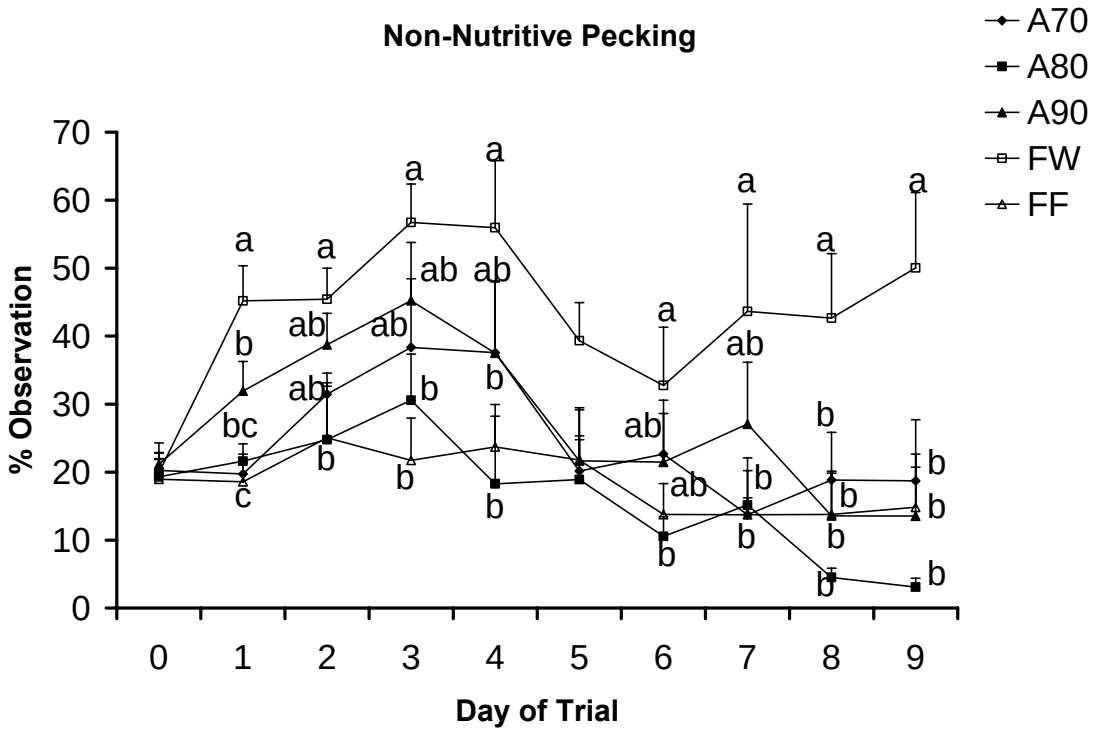


Figure 6.6: Percentage observations of nonnutritive pecking behavior of laying hens during a 9 day induced molt.
N= 6 hens per group. Treatments within the same day with different letters are significantly different at $P \leq 0.05$.

nonnutritive pecking behavior from the A90, A80, and FF hens. On d 8 and 9 the FW hens spent significantly more ($P \leq 0.0006$) time than the other treatments in nonnutritive pecking behavior.

Over the nine day period of feed withdrawal the FW hens spent significantly more time performing nonnutritive pecking than all the other treatment groups while the alfalfa treatment groups compared favorably to the FF hens. Among the alfalfa treatments the A90 hens spent most time performing nonnutritive pecking behavior, they were not significantly different from the A70 hens. Of the alfalfa treatments, the A80 hens spent the least amount of time performing nonnutritive pecking and were similar to the FF hens, while the A90 and A70 hens spent more time than the FF and the A80 hens. The increasing occurrence of nonnutritive pecking observed early in the trial in the alfalfa fed hens later subsided to occurrences similar to the FF hens, this could be because they did not adapt immediately to the change of diet. Nonnutritive pecking is thought to be redirected foraging behavior that is seen in birds in the wild and evidence shows that even when hens are provided with adequate food they are still motivated to forage (Duncan and Hughes, 1972). Petherick and Rutter (1990) stated that hens will act in a predictable manner to feed deprivation by working harder for food the longer they have been without it. Other reports in studies where hens were deprived noted that feed restricted chickens have exhibited increased nonnutritive pecking (Savory and Fisher, 1992; Savory et al., 1992; Webster, 2000; McCowan et al., 2006).

Feeder Activity

Significant ($P < 0.0004$) treatment by day effect was observed in feeder behavior (Figure 6.7) during this trial. On d 1 of the trial the A70 and A90 hens spent significantly more ($P \leq 0.006$) time at the feeder than the other treatments and they were not different from each other ($P \geq 0.8$). The A80, FF and FW hens did not differ ($P \geq 0.3$) significantly on d 1. No significant differences were observed among treatments on d 2. On d 3 the FF hens spent significantly more ($P \leq 0.03$) time at the feeder than the A80 and the FW hens. The A90 and A70 hens were not significantly different ($P \geq 0.05$) from any of the other treatments on d 3. On d 4 the A80 hens spent significantly more ($P \leq 0.001$) time at the feeder than the FW hens, no other significant differences were observed on this day. No significant differences were observed on d 5, however, on d 6, 7, and 9 the FW hens spent significantly less ($P \leq 0.0001$) time than the other treatments at the feeder, while the other treatments were not significantly different. On d 8 the A90, A80 and A70 hens spent significantly more ($P \leq 0.0001$) time than the FW hens at the feeder and they were not significantly ($P \geq 0.05$) different from the FF hens. The FF hens and FW hens did not display significant difference ($P \geq 0.0008$) at the feeder.

As was anticipated, the hens which had feed spent more time at the feeder than the FW hens. Since we could not tell whether or not a bird was eating, we scored each time the bird visited the feeding troughs and because of this the FW hens were sometimes given a score for feeding. At the beginning of the trial the A90 and A70 hens visited the feeder more often than any other group, however, from d 2 up to d 5 the

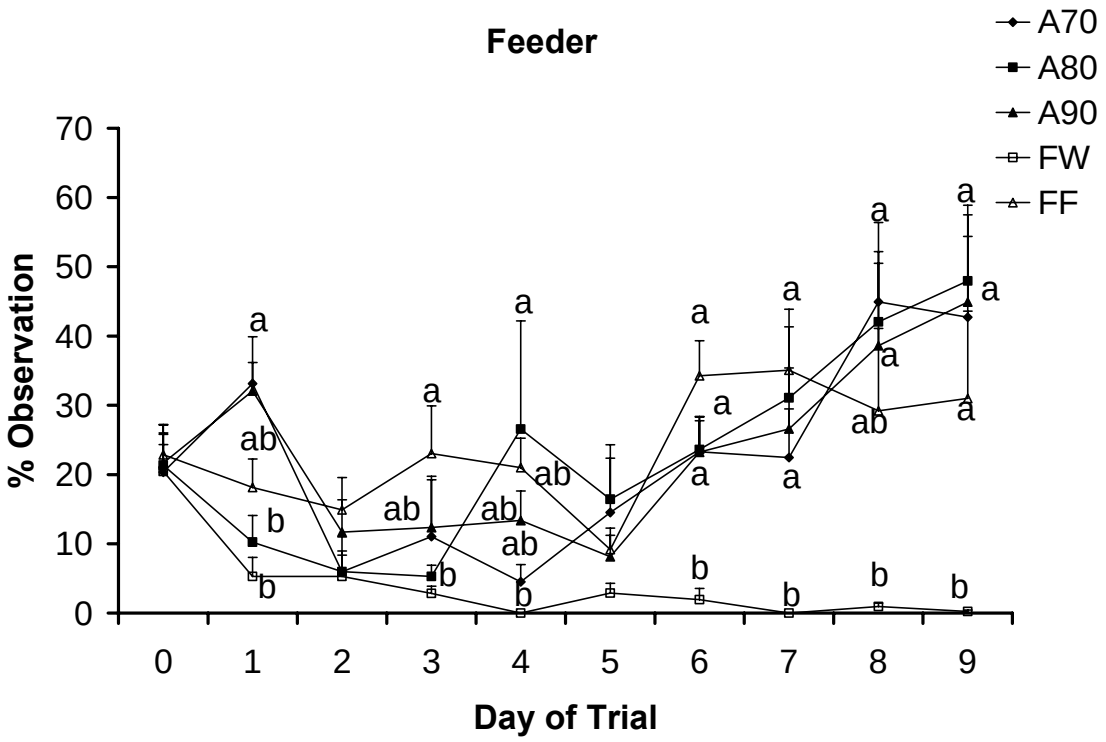


Figure 6.7: Percentage observations of feeder behavior of laying hens during a 9 day induced molt.
N= 6 hens per group. Treatments within the same day with different letters are significantly different at $P \leq 0.05$.

number of visits declined, after d 5, the A90, A80, and A70 hens all spent increasing amounts of time visiting the feeder and were not significantly different from each other for the duration of the trial. For the duration of the trial the A90 hens exhibited feeder behavior that was closest to that displayed by the FF hens. Of the alfalfa fed hens A70 hens displayed the least amount of time visiting the feeder and the A80 was intermediate. The reduction in visits to the feeder at the initial stages of the trial could have been an adjustment period for these hens to the change of diet. The reduced intake of the alfalfa diet early in the trial could also be due to the unpalatability of alfalfa because of the presence of saponins (Sen et al., 1998) or the slow passage of the alfalfa through the gastrointestinal tract of the chicken (Sibald, 1979, 1980). It has been demonstrated that hens fed alfalfa diets ate significantly less than hens fed layer ration (Woodward et al., 2005; Donalson et al., 2005). When Donalson et al. (2005) compared different ratios of alfalfa diets they observed the A70 hens ate more than the A90 hens. We also observed what appeared to be a relationship between feeder activity and nonnutritive pecking because as nonnutritive pecking declined in frequency, feeder activity increased but the reduction in frequency was not dependent on the treatment. Webster (1995, 2000) reported a similar relationship when molting hens by feed deprivation, they also stated that hens which were molted using feed withdrawal spent progressively less time visiting the feeder as the molt period progressed.

It has been implied that feed deprivation as a means of inducing a molt can result in reduced animal welfare, however, Webster (1995, 2000, 2003) has stated that there is

no evidence that hens deprived of feed for a period to induce a molt actually suffered during the molt induction or that they suffer long term effects from the induced molt.

Little behavior work has been done investigating the effects of alternative molt diets on laying hens. The results from this trial indicated that the alfalfa molt diets did not result in increased aggressiveness in the molting hens. The A90 and A80 hens stopped laying at a time that was comparable to the FW hens. The hens fed alfalfa- layer ration diets remained active and alert throughout the trial as indicated by their head movement and walking activities during the trial. The alfalfa fed hens spent less time drinking than the FF hens but this could be because they ate less than the FF hens. When comparing the alfalfa diets, the A70 hens spent the least amount of time drinking and the least amount of time at the feeder, on the other hand the A90 hens spent the most amount of time eating but the A80 hens drank more than them. At the beginning of the trial the hens fed alfalfa diets spent a similar amount of time performing nonnutritive pecking as the FW hens, however, this declined towards the end of the trial. Reduced nonnutritive pecking behavior was not observed in the FW hens and instead the behavior increased in these hens towards the end of the trial. With the reduction in nonnutritive pecking behavior, an increase in feeder related activity was observed, this indicated that the hens required some time to become accustomed to the new diet. The A90 hens compared least favorably with the FF hens in nonnutritive pecking behavior. The A80 hens appeared to be the closest to the FF hens and the A70 hens were also similar to the FF hen in nonnutritive pecking behavior. The use of different combinations of alfalfa diets to induce a molt have been explored (Donalson et al., 2005) and the A70 was the least

favorable due the incomplete regression of the reproductive tract, in this trial the A70 hens spent less time preening and began shedding at a later date than the other two alfalfa diets, also, they did not stop completely laying up to d 9. No adverse effects to the hens' welfare surfaced in the A90, A80, or A70 hens during this trial based on the behavior parameters we examined; in fact the diets effectively reduced some of the abnormal behaviors such as increased nonnutritive pecking which has been observed in feed deprived hens. Additional studies are necessary to examine physiological and immunological changes associated with stress in hens that are fed these diets to induce a molt.

CHAPTER VII

APPLICATION OF HAFNIUM CHLORIDE AS A MARKER TO ASSESS THE PASSAGE RATE OF LAYER RATION AND ALFALFA DIETS

Introduction

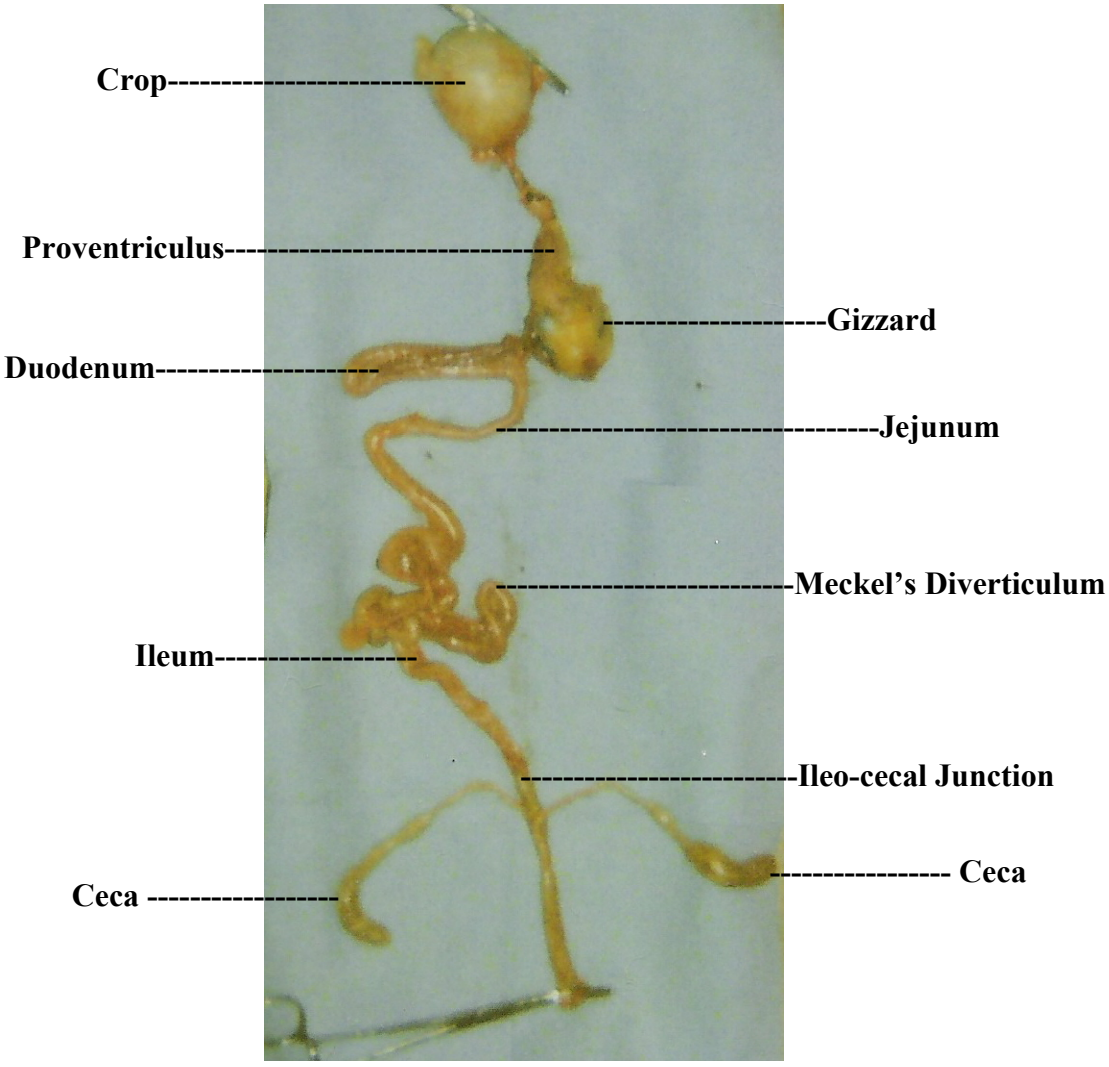
Molting in the avian species can be defined as the periodic shedding and replacement of feathers, and the rejuvenation of the reproductive system (Svihus et al., 2002). While the process occurs naturally in chickens, it is usually for a prolonged period of time during which the birds stop laying. This poses a problem for the commercial industry and a solution is to force molt the hens in an effort to prevent economic losses due to low egg production. Several diets have been developed which induce molting without resorting to conventional feed deprivation which has been strongly opposed by animal welfare groups (Wakeling, 1978; Shippee et al., 1979; Arrington et al., 1967). Landers et al. (2005) have shown that alfalfa can effectively induce a molt and Woodward et al. (2005) confirmed that alfalfa molt diets limited infestation of *Salmonella* in laying hens.

The effectiveness of molt rations is determined to a large extent by the rate of passage of the feed through the gastrointestinal (GI) tract and the length of time the feed stays in the various GI tract regions. Therefore the evaluation of various molt diet programs is dependent on the development of methods to study these digestion kenetics. Using indigestible flow markers to mark feed ingredients and then collecting the excreta at predetermined times is a common means of obtaining passage rate. Tukey et al (1958) observed that in chickens, markers first appeared in the excreta within 2 to 2.5 h after

intake and that most of the marker was usually excreted within 12 h. The use of indigestible marker methods requires that the marker dose is administered under steady state conditions and that the marker itself is impervious to GI tract conditions, especially acidity. Tetravalent hafnium (Hf) appears to bind with bonds that are very resistant to proton displacement, but this has not been demonstrated in chickens.

Feed passage rate can vary in different segments of the GI tract and is affected by the composition of the feed. To measure the passage time in each compartment of the tract Van der Klis and Van Vorst (1993) fed birds and killed them at different time intervals, then carefully dissected them and removed the contents of each compartment to analyze. This method of measuring mean residence time of the rations is less sensitive to non-steady state conditions, but still requires indelible flow markers.

The purpose of the present work was to evaluate the potential for using Hf as a stable isotope indigestible marker and its application to measurements using the methods mentioned. To this end we administered three differing marked diets to groups of laying hens, collected fecal material at selected intervals and collected digesta from various portions of the GI tract (Figure 7.1) following the sacrifice of the birds. We then measured the neutron activation analysis determination limit for hafnium in the applicable fecal material and digesta, determined optimum concentration for dosing solutions, and performed preliminary passage rate studies in laying hens.



Picture by P. Herrera and C. Dunkley (2004)

Figure 7.1. Picture of the hens' gastrointestinal tract. Ingesta were removed from the Crop, Gizzard, Ileun, Jejunum, and Ceca.

Materials and Methods

Experimental Design

A trial used Single Comb White Leghorn hens over 60 weeks old that were obtained from a local commercial laying flock. The hens were placed in wire layer cages and were given free access to water and a layer ration that met the National Research Council recommendations for nutrients (NRC, 1994). They were allowed to acclimatize in the cages for a period of two weeks after which the photo-period was changed from 16 h light: 8 h dark, to 8 h light: 16 h dark. One week after the light change the hens were randomly assigned to three groups. There was a non-molted group which was used as the control group and was given layer corn ration throughout the study. The other two groups were molted using two different alfalfa diets: 100% alfalfa and 90% alfalfa plus 10% layer ration. Each group consisted of 10 hens. One week after the change of lights, the alfalfa groups were given unmarked molt diets, indicating the first day of the molt. On the third day of the molt, the feed was removed from the hens in all three groups for a period of approximately three hours, and then replaced with marked ration. The diets were marked according to the procedure described by Worley et al. (2004). Feed components were allowed to soak in a solution of ammonium hafnyl carbonate solution containing about 16.4 mg Hf/ml for 12 h, rinsed twice with distilled water and used in the formulation of the meals. The hens were allowed to consume the meals containing Hf marked corn and/or alfalfa for one hour after which it was then removed and replaced with unmarked diets. Fecal droppings were collected 1, 2, 4, 6, 8, 10 and 24 h after the marked feed was removed from the hens. The droppings were

dried, weighed, homogenized and packaged into pre-cleaned polyethylene vials for analysis.

The marked meals were once again administered to the hens for 1 h on the sixth day of the molt. Half (n=5) of the hens in each of the groups were slaughtered after two hours and the other half were slaughtered 1 h after the marker was reintroduced. Feed consumption was recorded and residual feed weighed to compute marker dose intake. Digesta was taken from the crop, the gizzard, the jejunum, the ileum and the ceca, as described by Svihus et al. (2002). Care was taken not to include lining of mucosa and only undigested feed was collected. Digesta samples were then dried, homogenized and packaged for neutron activation analysis. The mass of material collected from each digestive compartment was recorded.

Neutron Activation Analysis

The neutron activation protocol used in this study has been developed and applied in several earlier animal studies (James et al., 1983; Pond et al., 1985; Wylie et al., 2000). In brief, samples were prepared by encapsulation in pre-cleaned polyethylene irradiation vials. Comparator standards were prepared by evaporation of weighed quantities of primary standard solutions onto cellulose. The quantity of cellulose was varied in such a way to allow the physical geometry of the standards prepared to match those of the digesta samples analyzed. Approximately 10µg of hafnium in 100µL solution was deposited for each standard prepared. Samples and standards were then irradiated together in the Texas A&M Nuclear Science Center's 1MW research reactor facility for 3 hours at a nominal neutron flux of $1x10^{13}$ $cm^{-2}s^{-1}$ in a rotisserie position. All

irradiated materials underwent gamma spectroscopy after a decay periods of about one week. Spectra were accumulated for 30 min on a Canberra Industries Alpha-based VMS Genie system using high purity germanium detectors from Canberra or Ortec/Ametek. The 482 keV gamma line from ^{181}Hf was utilized for comparison of samples to standards and computation of hafnium content was accomplished using Canberra's Genie NAA software.

Results and Discussion

Limits of detection were determined for hafnium in each of the sample matrices and are presented in Table 7.1. In every case the sensitivity of the measurement proved to be sufficient, with all detection limits falling far below the targeted concentrations to reveal presence of marker. The concentrations of hafnium in fecal droppings from hens fed the control layer ration diet showed a distinct passage peak. Levels varied from initial values averaging some 18 μg/g up to a maximum of approximately 460 μg/g after about 4 h following the administration of the marked meal. The variation of results from individual chickens within the group was very large. Figure 7.2 presents plots of fecal hafnium content for each individual bird showing this disparity as well as the variation in time at which the maximum concentration appeared. One could also note from the figure the small secondary maximum occurring 8 h after the dose for several of the hens. Perhaps more instructive, Figure 7.3 presents the averages of the group. From this graph it is observed that only a small portion of the marker is present in feces and the average maximum occurs between the 2nd and 4th hour post-dose.

Table 7.1: Hafnium detection limits in sampled matrices

Sample Matrix[1]	Average Mass (mg)[2]	Hafnium (ng/g)[3]
Feces (n=6)	430	26
Digesta from Crop (n=4)	660	12
Digesta from Gizzard (n=4)	430	22
Digesta from Jejunum (n=4)	560	48
Digesta from Illeum (n=4)	520	36
Digesta from Ceca (n=4)	560	57

[1]Sample taken from the feces and segments of the digestive tract
[2]Average mass of the sample in mg
[3]Concentration of hafnium in samples

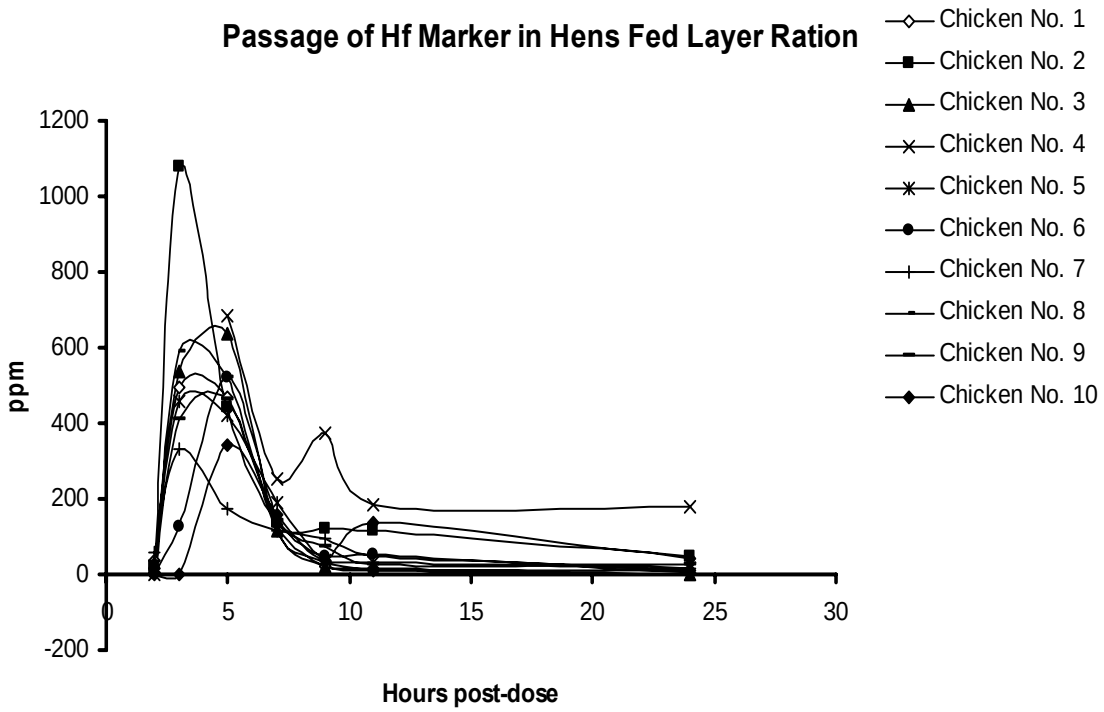


Figure 7.2: Plot of hafnium concentrations in feces from the individual hens, as a function of time after administration of corn-soy based layer ration marked meal.

By the 10th hour post-dose the majority of the marker was excreted even though marker was observed 24 h after the hens were dosed. Similar results were observed by Tukey et al. (1958).

Although identical procedures were used for the absorption of the marker onto the corn ration layer diet and the alfalfa diets, only minimal marker was detected in the fecal droppings from hens fed alfalfa meals after 1 h delay. Clearly the binding of the Hf to the alfalfa was far less efficient than to the corn diet and than was expected to alfalfa, or the element was primarily released from the marked meal within the GI tract. In any case, the majority of the marker in alfalfa-fed hens was present in the initial fecal collection at 1 h post-dose. As a result passage rate curves are not possible from the data in this study. Application of hafnium marker technology to molt ration evaluation will depend on successful binding with meal diets.

Figure 7.4 to 7.6 presents data from the GI tract compartment study. Once again the concentration averages of Hf in digesta from all of the compartments are more than an order of magnitude greater when the marker is associated with corn ration than with the alfalfa diets. However, although minimal absorption of the marker is evident with the alfalfa diets, one can see a very similar pattern among Hf concentrations in the various organs for all three treatments corn-soy based layer rations. Even after only 2 h, the marker is found predominantly in the ileum of the hens from the alfalfa 100 and the alfalfa 90. After 7 h the concentrations are reduced but retain the similar pattern.

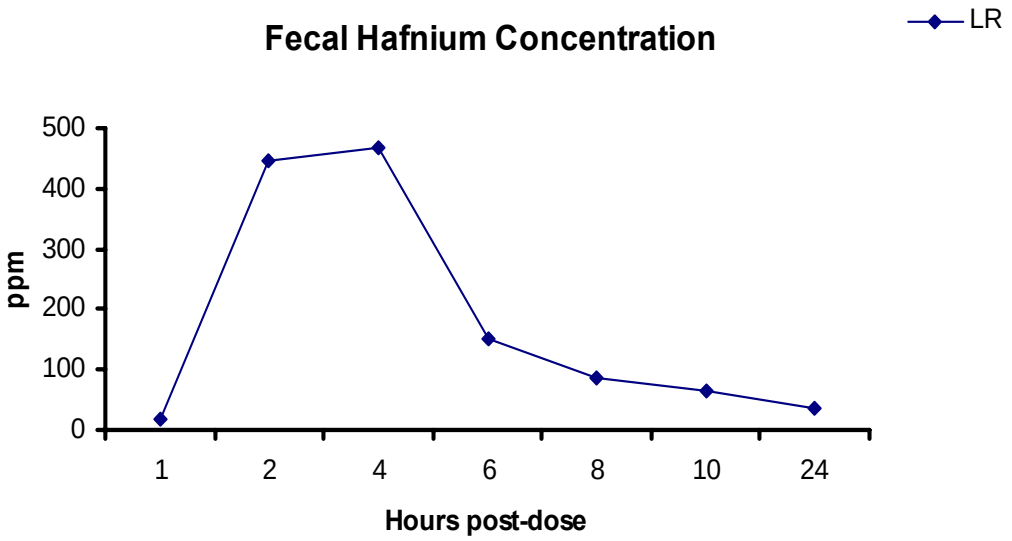


Figure 7.3: Plot of hafnium concentrations in feces from the hens, as a function of time after administration of corn-soy based layer ration marked meal.

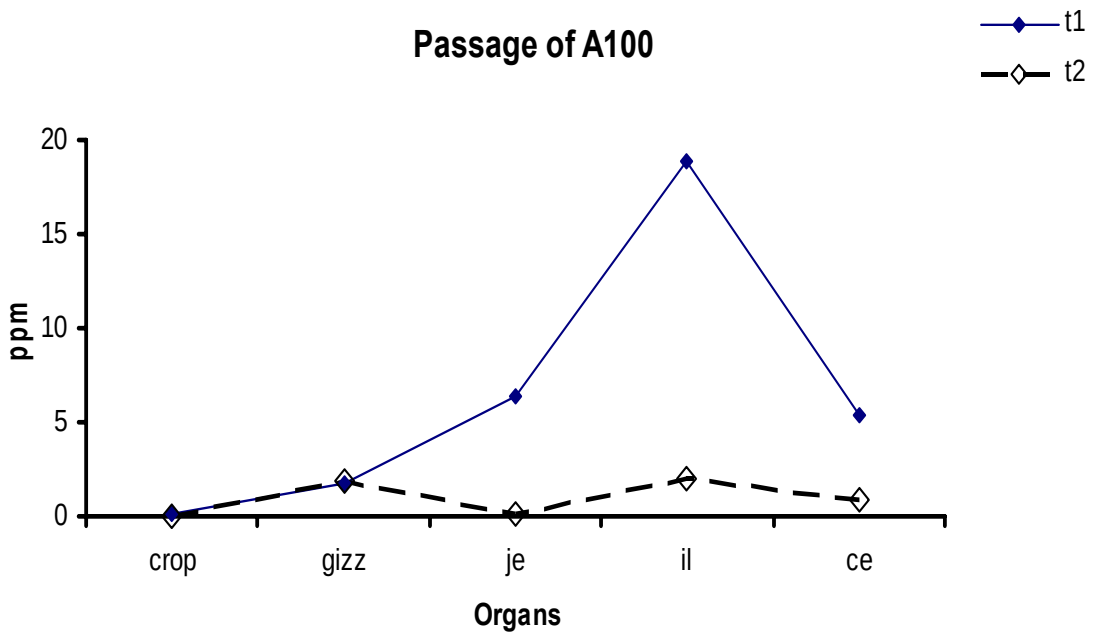


Figure 7.4: Concentration of the Hf marker present in the alfalfa 100 diet in different segments of the hens' digestive tract at 2 hours and 7 hours post-dose. Crop- crop of tract, gizz- gizzard of tract, je-jejunum of tract, il- ileum of tract, ce- ceca of tract.

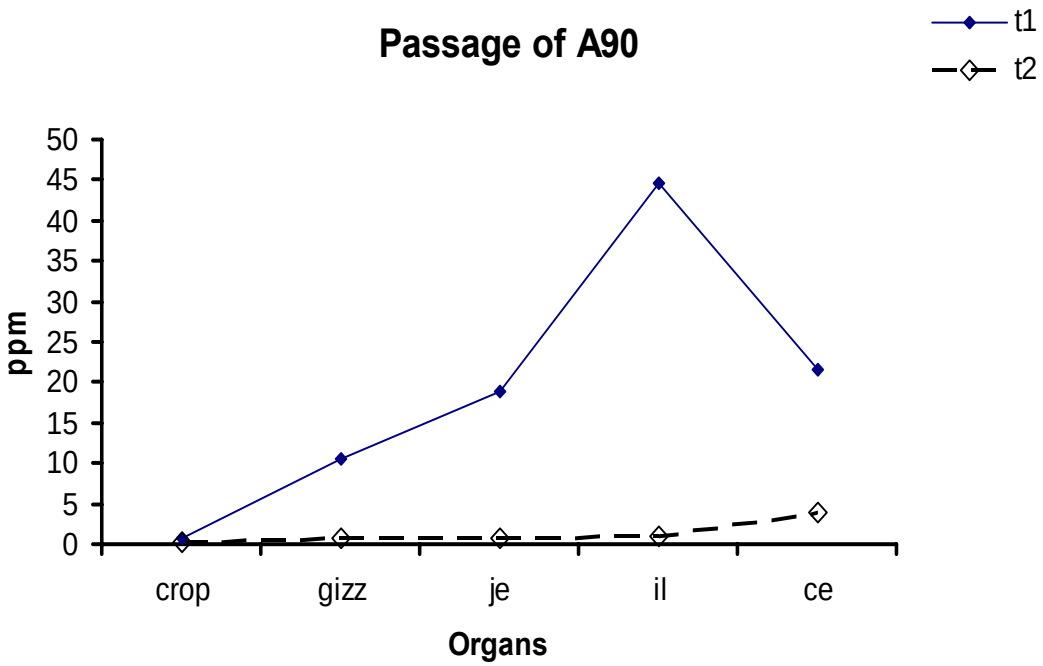


Figure 7.5: Concentration of the Hf marker present in the alfalfa 90 diet in different segments of the hens' digestive tract at 2 hours and 7 hours post-dose. Crop- crop of tract, gizz- gizzard of tract, je-jejunum of tract, il- ileum of tract, ce- ceca of tract.

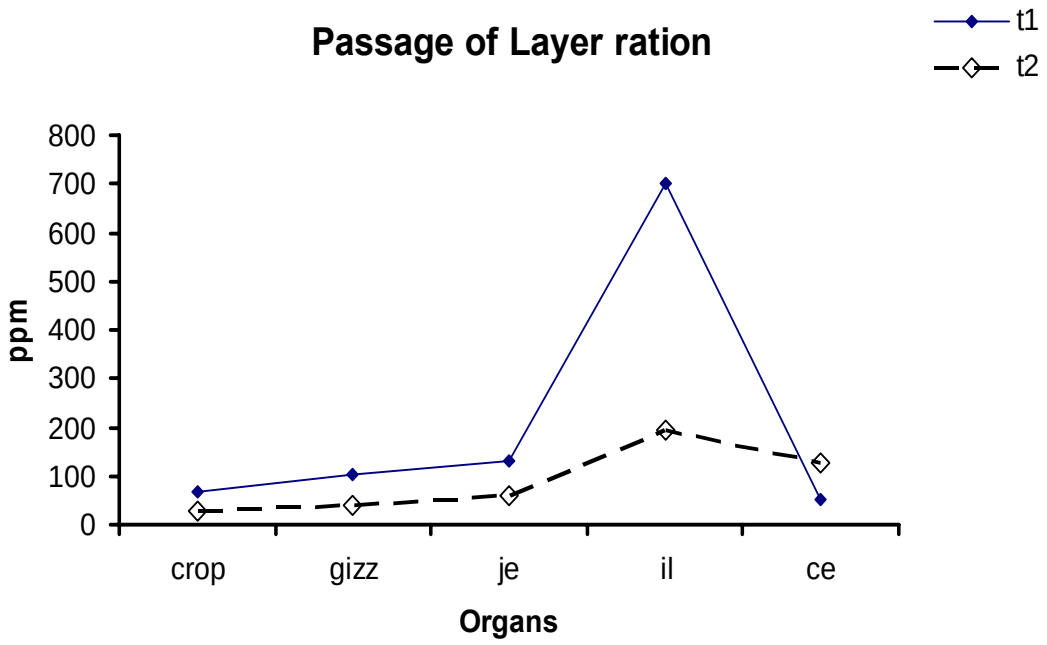


Figure 7.6: Concentration of the Hf marker present in the layer ration in different segments of the hens' digestive tract at 2 hours and 7 hours post-dose.
Crop- crop of tract, gizz- gizzard of tract, je-jejunum of tract, il- ileum of tract, ce- ceca of tract.

In conclusion the use of hafnium as a stable isotope marker has been shown useful for the study of digestion processes in chickens. The absorption of the marker element onto the corn meal component using our methods was shown to be very efficient. Passage curves constructed from the data are in good agreement with previous work. Inspection of GI tract compartments of the hens revealed that the majority of the marker was in the ileum by 2 h post-dose. Little marker element was found in digesta or feces following dosing of alfalfa diets. Either absorption onto the alfalfa meal component was minimal or the element was rapidly released from the alfalfa in the GI tract of the birds. The is likely because the levels of Hf in the ingesta of the A90 hens were much higher than what was observed in the A100 hens therefore the Hf was bound to the layer ration that was included in the A90 diet. However, inspection of GI tract compartments still showed a maximum concentration in the ileum, although at significantly lower concentrations than for corn-soy based layer ration fed hens. Additional experiments are required to resolve this issue before the method can be used to further study digestion kinetics of alfalfa molt diets.

CHAPTER VIII

SUMMARY AND CONCLUSIONS

Molting is a phenomenon that occurs naturally in birds usually at the end of a breeding season in the case of a reproductive molt. In order to obtain a second laying season from their hens, egg producers will induce a molt in their flock by altering the day length and withdrawing feed from the hens for a maximum period of 14 days. It has been shown that in laying hens feed deprivation causes the suppression of the immune system and they become susceptible to gastrointestinal pathogens such as *Salmonella*. Abnormal behaviors such as increased nonnutritive pecking, stereotypy and increased aggression have also been observed in feed deprived hens. Concerns of reduced animal welfare have led to increased criticism of the practice of feed deprivation as a method to induce a molt. There is now a need for the development of an animal friendly molting practice that will alleviate reduced welfare associated with feed deprivation. The two components of animal welfare are physiological and psychological health, therefore knowledge and understanding of the method that will be incorporated as a replacement of feed deprivation is necessary in order to prevent more harm than what would have occurred from a fasting method.

Previous research have shown that alfalfa diets can successfully induce a molt in a time that is comparable to feed derived hens, the regression of the reproductive tract, and hens that were molted using alfalfa diets exhibited comparable post-molt production parameters to that of feed deprived hens. In this research, physiological, immunological and behavioral aspects of using alfalfa diets were examined.

The effectiveness of alfalfa to reduce stress was determined by examining the blood plasma metabolites. The alfalfa fed hens had serum triglyceride levels that were similar to the feed deprived hens, which can be an indication that the ovaries were regressed. Alfalfa molted hens had total protein levels that were similar to what was observed in the feed deprived and full-fed hens. Uric acid production is an indication of protein utilization and the hens fed alfalfa had increased levels of uric acid similar to the full-fed hens by the end of the molt. The alfalfa fed hens did not display elevated levels of cholesterol or glucose that are associated with increased stress.

When evaluating the immune response of hens fed alfalfa diets, we observed that the serum antibody response of the hens on the alfalfa diet was comparable to the full-fed hens throughout the molt period, while the feed deprived hen first had a reduced antibody response at the beginning of the molt. The H: L ratio of the hens that were fed alfalfa during the molt period was similar to that of the full-fed hens while the feed deprived hens had elevated H: L ratios. The intestinal antibody secretion of hens fed an alfalfa diets and inoculated with SE was comparable to that of the full-fed hens. The acute phase response is a good indicator of inflammation, when we determined the acute phase response of hens fed an alfalfa diet and inoculated with SE during a molt we observed a response which was more than the full-fed hens but less than the feed deprived hens at the beginning of the trials, but by the end of the molt period these levels were reduced to levels that were similar to the full-fed hens.

The results from the behavior studies showed that the hens fed alfalfa during the trials did not display increased incidences of aggression during the trial period. The

increased nonnutritive pecking behavior that was displayed during the molt period by the feed deprived hens was also observed in the hens in the alfalfa treatment group early in the molt however, this behavior steadily declined during the molting period to levels that were comparable to the full-fed hens. We also noted that the feeder activities of the alfalfa fed hens increased over the molt period to levels that were similar to the full-fed hens. The walking activities of the alfalfa fed hens did not decline over the molt period and they spent equal amount of time involved in head movements as the full-fed hens. There was increased preening activity displayed by the alfalfa fed hens and the feed deprived hens as the period of molt progressed, which could be used as an indication of integumental irritation and these hens started shedding their feathers on days 10 and 8 respectively.

We observed that hafnium chloride could be useful to track the passage of corn-soy ration through the gastrointestinal tract of the chicken. It is possible that the rare earth did not effectively mark the alfalfa or it became dissociated from the diet in the gastrointestinal tract of the hens, this was also evident when we did not detect acceptable levels of the marker in the feces of the alfalfa fed hens.

Based on the results from these studies, alfalfa can reduce physiological and psychological harm to laying hens while on an induced molt program. Accordingly, alfalfa can be effectively implemented as an alternative diet for molt induction that will help to resolve some of the concerns of reduced animal welfare during molt induction by feed deprivation.

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